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EXOGENOUS ADMINISTRATION OF VISFATIN AFFECTS CYTOKINE SECRETION AND INCREASES OXIDATIVE STRESS IN HUMAN MALIGNANT MELANOMA ME45 CELLS

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Visfatin has recently been established as a novel adipokine that is predominantly expressed in visceral fat. Recombinant visfatin has immunomodulating properties, which can activate human leukocytes *in vitro* to induce cytokine production (IL-1 β , TNF- α , and IL-6). Only few studies have investigated the effect of visfatin on prostate, breast, ovarian cancer as well as astrocytoma cell biology. There have been no studies on the cytokine secretion in human melanoma cells in response to visfatin stimulation along with intracellular protein kinases inhibitors. ELISA assay was performed in supernatants of Me45 cells stimulated with visfatin in the presence or the absence of specific pharmacological inhibitors of the indicated protein kinases (p38, MEK 1, PI3k and JAK kinase) and nuclear factor kappa B (NF- κ B) inhibitor. Intracellular reactive oxygen species level was measured in 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA)-loaded cells using a fluorescent measurement system. For determination of NF- κ B activation, activated NF- κ B p65 subunit was determined using an EZ-TFA-detect chemiluminescent transcription factor assay. We report that visfatin led to the significant increase in IL-6 and IL-8 level in culture supernatants of human malignant melanoma Me45 cells. Additionally visfatin resulted in the increase of the intracellular reactive oxygen species level. PI3k and NF- κ B pathways were activated upon visfatin stimulation. The results may reflect the fact that PI3k pathway stimulation by visfatin may further lead to NF- κ B activation and pro-inflammatory response.

Key words: *visfatin, cytokines secretion, nuclear factor kappa B activation, reactive oxygen species, melanoma*

INTRODUCTION

Visfatin has been strongly associated with the systemic inflammation and, to a lesser extent, with the oxidative stress; nevertheless the causal relationships between these factors remain unclear (1).

Data regarding visfatin concentration in people suffering from carcinomas are scarce, however available in the literature (2-4). In healthy subjects, circulating concentration of visfatin are significantly increased in obese subjects (up to 2.5 ng/ml) compared to lean controls (up to 1 ng/ml) according to ELISA assay (5). In other cases, plasma visfatin levels in women with polycystic ovary syndrome (PCOS) are (30.2 ng/ml \pm 10.4) when compared to normal controls (11.2 ng/ml \pm 10.4) according to RIA assay (6). Zwirska-Korczala *et al.* (7), reported increase in level of serum visfatin in obese PCOS women (24.14 ng/ml \pm 4.07) when compared to lean PCOS subjects (17.2 ng/ml \pm 5.13) and healthy controls (14.2 ng/ml \pm 2.3). Moreover, serum visfatin levels are significantly higher in postmenopausal breast cancer cases (57.9 \pm 31.3 ng/ml) than in control subjects (43.6 \pm 28 ng/ml) and patients with benign breast lesions (42.9 \pm 18.1

ng/ml) (2). This is in agreement with other studies reporting significantly higher mean serum visfatin levels in gastric and colon cancer patients than in age- and gender-matched controls (3, 4). Serum visfatin was also reported as a good biomarker of colorectal adenocarcinoma malignancy potential and stage progression (3). However data on serum visfatin level in patients with mesenchymal malignant neoplasms are lacking.

Chronic inflammation also has been linked to various steps involved in carcinogenesis, including cellular transformation, oxidative stress, infiltration, proliferation, angiogenesis and metastases (8-10). Visfatin is a novel fat derived adipocytokine, secreted by visceral and subcutaneous fat tissues (11, 12), hepatocytes (13), monocytes and macrophages (14-16). Visfatin has both intra- and extracellular forms in mammals (17). The extracellular form of this protein has been reported to act as a cytokine named visfatin/PBEF (18), an insulin-mimetic hormone (19), or an extracellular NAD biosynthetic enzyme named visfatin/eNampt (20). Only few studies have investigated the effect of visfatin on cancer cells *in vitro* and *in vivo*. Increased expression of visfatin was confirmed in ovarian cancer (21), malignant astrocytoma (22), human prostate cancer cell

lines; LNCaP (androgen-sensitive) and PC3 (androgen-insensitive) as well as human prostate cancer (23). Our previous findings suggest that visfatin triggers a redox adaptation response, leading to an up-regulation of antioxidant capacity in Me45 melanoma cells. Visfatin led to a significantly increased proliferation rate in the study using the [3H]thymidine incorporation method in these cells. Unlike insulin, visfatin-induced melanoma cell proliferation was not mediated by an insulin receptor (24).

Aims of the study: (i) determination of the influence of visfatin on cytokine secretion (IL-1 β , IL6 as well as on IL-8) in human malignant melanoma Me45 and human dermal fibroblasts NHDF cell lines; (ii) evaluation of the secretion of these cytokines by Me45 cells stimulated with visfatin in the presence of specific pharmacological inhibitors of protein kinases (MEK [PD 98059], PI3k (LY 294002), p38 kinase [SB 203580], JAK inhibitor I [DBI] and NF- κ B inhibitor [wedelolactone] (iii) assessment of the influence of visfatin on NF- κ B activation in Me45 melanoma cells.

MATERIALS AND METHODS

Cell culture

A human malignant melanoma Me45 cell line was obtained from the Silesian University of Technology, as a kind gift from dr M. Widel from the Marie Curie Memorial Cancer Centre and Institute of Oncology, Gliwice, Poland. The cells were derived from metastatic lesions (local lymph nodes) of a 35-year-old patient of the Institute of Oncology (25). A human dermal fibroblast NHDF cell line was purchased from the PromoCell. Those cells were isolated from the human dermis of juvenile foreskin.

Me45 melanoma cells were plated at the density of 1×10^6 cells per 25 cm² flask and cultured in the Dulbecco's Modified Eagle's Medium with L-glutamine (Sigma-Aldrich, St Louis, Mo) supplemented with 10% fetal bovine serum (Gibco, North Andover, Mass), antibiotics: penicillin (10,000 I.U./ml), streptomycin (10000 μ g/ml), amphotericin B (2.5 μ g/ml) (Sigma-Aldrich, St Louis, Mo) under the atmosphere of 95% air and 5% CO₂ at 37°C. NHDF fibroblasts at the density of 1×10^6 cells per 25 cm² were cultured in ready to use fibroblast growth medium obtained from PromoCell (cat no. C-23010) and were supplemented and cultured in the same manner like above mentioned melanoma cells. Me45 and NHDF cell lines were free of mycoplasma, pathogenic viruses and bacteria. Cultures were maintained for no longer than four weeks after recovery from the frozen stock.

Visfatin (Alexis Biochemical, Plymouth, PA) was dissolved in PBS without Mg²⁺, Ca²⁺ (Sigma-Aldrich, St Louis, Mo). The solutions were prepared fresh, protected from light, and added to the incubation medium at the following final concentrations: 10 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml. The purity of visfatin was 96-97% (SDS-PAGE analysis) and contained <0.01 ng/ μ g LPS as determined by the *Limulus* amoebocyte lysate method.

ELISA assay for cytokines

Human malignant melanoma Me45 and non-malignant NHDF fibroblasts cell lines were seeded at the concentration of 1×10^5 /ml in the appropriate medium described above. After 24 h, the medium was removed and the cell culture was supplemented with fresh serum-free medium. Cells with the serum-free medium were treated with different doses of visfatin 10–250 ng/ml for 6 and 24 hours. Additionally, we also tried to examine the effects of specific protein kinase inhibitors on cytokines

secretion after 24 hours. In this study experiment, cells were stimulated with 100 ng/ml visfatin in the presence of a specific pharmacological inhibitor of the indicated protein kinases (MEK 1 [PD 98059], PI3k (LY 294002), p38 kinase [SB 203580], JAK inhibitor I [DBI], all protein kinase inhibitors were purchased from Calbiochem, EMD Biosciences. NF- κ B inhibitor [wedelolactone] was purchased from Sigma-Aldrich.

After 6 and 24 hours, the medium was centrifuged (2000 rpm for 5 min) and supernatants were harvested and stored at -20°C and afterwards tested for cytokines with ELISA. The concentrations of IL-1 β , IL-6 and IL-8 in cell culture supernatants were determined using commercially available ELISA kits, according to the manufacturer's instruction. The absorption was determined with the iMark microplate absorbance reader at 450 nm (BioRad). The lower detection limit was 0.3 pg/ml for human IL-1 β (coefficients of variation; intra-assay: 6.3%, inter-assay: 8.7%) and 0.92 pg/ml for human IL-6 (coefficients of variation; intra-assay: 3.4%, inter-assay: 5.2%), and 2 pg/ml for human IL-8/NAP-1 (coefficients of variation; intra-assay: 6.3%, inter-assay: 8.7%). The ELISA kits were obtained from BenderMedSystem, Austria.

Determination of activated NF- κ B p65 (RelA)-binding activity

For determination of NF- κ B activation, 2×10^6 Me45 cells per ml were incubated with or without visfatin (100 ng/ml). Total nuclear and cytosol protein was extracted with M-PER protein extraction reagent (Pierce Protein Biology Products) in the presence of a protease inhibitor mixture (Sigma-Aldrich) after 0, 1, 3, 6, 10, 12, 24 and 48 hours. Protein concentrations were determined by the Bradford protein assay (Bio-Rad). Activated NF- κ B p65 was determined using an EZ-TFA-detect chemiluminescent transcription factor assay (Merck, Milipore). Briefly, 10 μ g of total protein was incubated in wells containing biotinylated-consensus DNA duplexes of NF- κ B. The captured active transcription factor was detected by a specific antibody (Ab) recognizing NF- κ B p65 and then incubated with a secondary HRP-conjugated Ab. A chemiluminescent substrate was added to each well and the resulting signal was measured using a GloMax microplate luminometer (Promega) according to manufacturer's instruction.

Intracellular reactive oxygen species evaluation

Intracellular reactive oxygen species were detected in 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA)-loaded cells (Molecular Probe, Leiden, the Netherlands) using a fluorescent measurement system (Model Astroscan Cytofluor 2300/2350, Applied Biosystems, Millipore, Billerica, MA). Samples of 2×10^6 Me45 melanoma cells were placed on Corning 6-well plates (Sigma-Aldrich) and were pre-incubated with 5 μ M H₂DCF-DA for 1 hour at 37°C. The plates were centrifuged at 1200 rpm for 10 min and the fluorescence of control and treated cells was read in the Cytofluor reader (excitation at 504 nm, emission at 526 nm). The background of deacetylated, oxidised DCF (2', 7'-dichlorofluorescein) was approximately 65-75 relative fluorescent units/ (RFU) (26).

Statistics

The normality of distribution was evaluated by means of Shapiro-Wilk's test. All results are presented as a mean \pm S.D. Results have undergone statistical analysis, applying the one-way ANOVA test with the Bonferroni's post-hoc test or Kruskal-Wallis with Mann-Whitney U test, accordingly to parameter distribution. Changes at the level of significance of $p < 0.05$ have been assumed as statistically significant.

RESULTS

Visfatin induces IL-6 and IL-8 cytokine secretion in human malignant melanoma Me45 cells but not in human non-malignant NHDF fibroblasts

Human malignant melanoma Me45 cells did not secrete IL-1 β to the culture medium, regardless whether visfatin was in the cell culture (data not shown). However an increase in the secretion of the pro-inflammatory cytokines was observed. IL-6 and IL-8 concentration was elevated in a concentration- and time-dependent manner in the culture medium of Me45 cells after addition of visfatin (Fig. 1).

In the group treated with visfatin at 250 ng per ml for 6 h, a major increase in the IL-6 level was observed in comparison to the untreated group (21.35 ± 0.39 pg/ml versus 6.78 ± 0.23 pg/ml; $p < 0.05$, respectively), (Fig. 1A). Furthermore, IL-6 concentration after incubation with different concentrations of visfatin for 24 hours was higher when compared to medium

obtained from cell cultures treated for 6 hours. The highest secretion of IL-6 to the culture medium of Me45 cells was observed in the group treated with visfatin at 50 ng/ml upon 24 hours of incubation compared to the control group (43.84 ± 3.26 pg/ml versus 16.82 ± 1.21 pg/ml; $p < 0.05$, respectively), (Fig. 1B). The IL-8 level was significantly elevated in the group treated with visfatin at the concentration of 250 ng per ml after 24 hours in comparison to the untreated group (4553.43 ± 86.34 pg/ml versus 2445.21 ± 68.02 pg/ml; $p < 0.05$), (Fig. 2B). Regardless of the concentration used in the experiment the IL-8 secretion was slightly reduced after visfatin at 6 h (Fig. 2A).

As a physiological control we used human fibroblast NHDF cells that were stimulated with visfatin in the same culture conditions as melanoma cells. In contrast to melanoma cells, fibroblasts secreted IL-1 β cytokine to the culture medium (data not shown). We did not observe any statistical significant changes in visfatin-stimulated fibroblast cells compared to the untreated fibroblasts. Moreover, human NHDF fibroblasts produced almost 100-fold more IL-6 compared to melanoma

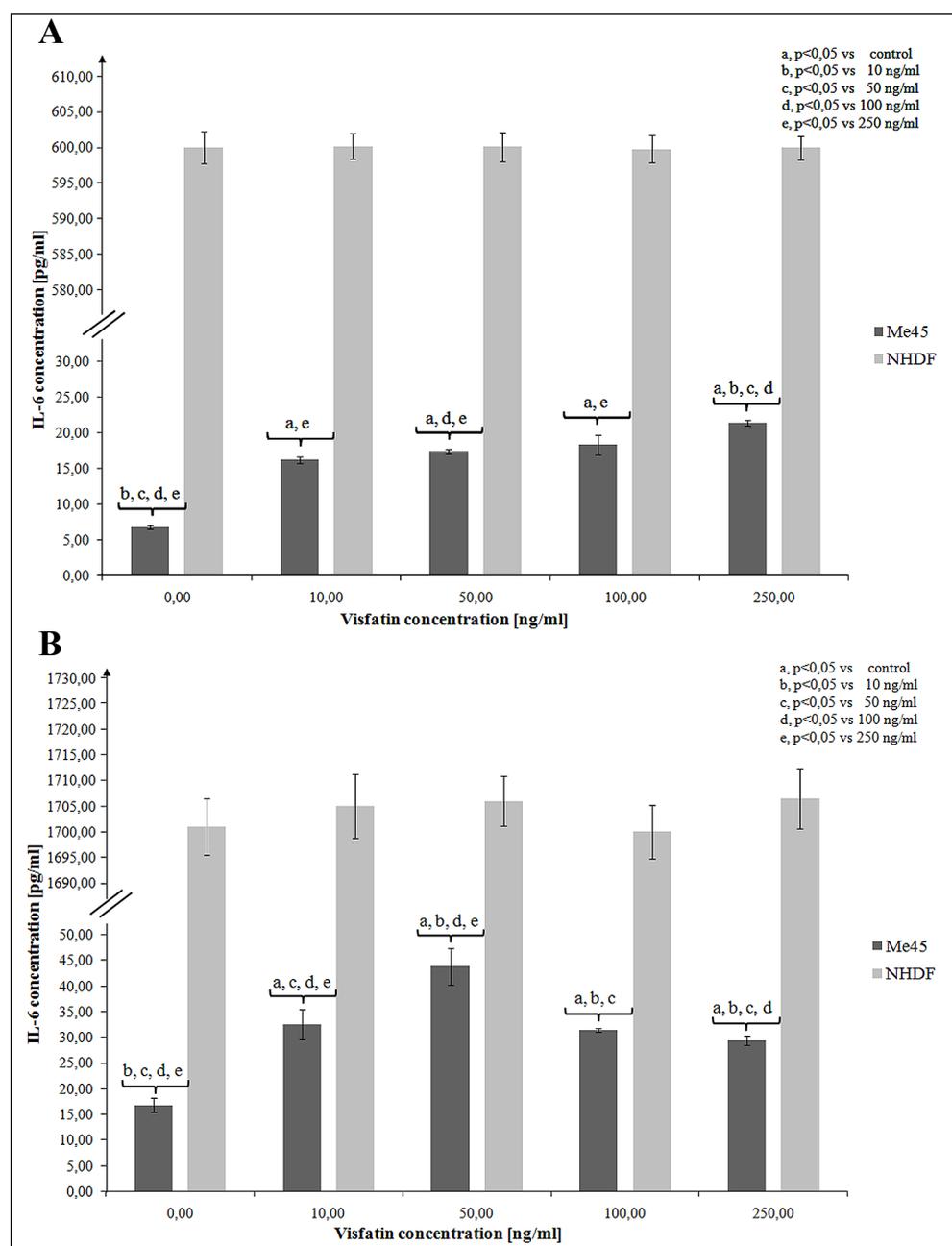


Fig. 1. IL-6 secretion in culture of melanoma Me45 cells and human fibroblasts after visfatin treatment 6 h (A) and 24 h (B) as determined by specific ELISA assay [pg/ml].

Me45 cells. This effect was even more pronounced after incubation for 24 hours (Fig. 1B).

Protein kinase inhibition, NF- κ B activation and reactive oxygen species generation after visfatin treatment

Treatment with inhibitors of different signaling pathways can provide useful information regarding intracellular pathway linkage and signal transduction in Me45 melanoma cells. We tested whether treatment with inhibitors against these pathways led to a decrease in visfatin-induced cytokine production in Me45 melanoma cells. PI3k inhibitor (LY294002) significantly reduced visfatin-induced IL-6 and IL-8 secretion. A significant rise in IL-6 (31.49 ± 0.63 pg/ml versus 16.82 ± 0.93 pg/ml; $p < 0.05$, respectively), (Fig. 3A) and IL-8 level (4051.99 ± 149.50 versus 2445.99 ± 67.53 pg/ml; $p < 0.05$, respectively) was observed in visfatin-treated Me45 cells (Fig. 3B). In the third group Me45 cells were treated only by specific pharmacologic inhibitor of PI3k, thereupon the level of studied

IL-6 has slightly increased with regard to un-treated Me45 cells (17.21 ± 1.09 pg/ml; $p < 0.05$ versus 16.82 ± 0.93 pg/ml; $p < 0.05$, respectively). Addition of PI3k inhibitor to cell culture medium slightly reduced level of IL-8 with regarding to control cells (2402.00 ± 61.28 versus 2445.99 ± 67.53 pg/ml; $p < 0.05$, respectively). After addition to Me45 cells both compounds, visfatin and PI3k inhibitor, an increase in IL-6 and IL-8 concentration was observed. However, the increase of IL-6 (21.53 ± 0.74 versus 31.49 ± 0.63 pg/ml; $p < 0.05$, respectively) and IL-8 (3390.00 ± 74.65 versus 4051.99 ± 149.50 ; $p < 0.05$, respectively) concentrations were not as high as visfatin-treated Me45 cell culture. Blockade of Janus tyrosine protein kinase activity JAK 1-3 (JAK/STAT pathway), inhibition of the p38 MAPK by SB203580 and MEK1 (MAP2K) through PD 98059 did not significantly prevented the production of IL-6 and IL-8 cytokines (data not shown). These observations may indicate indirectly the lack of central role for p38 MAPK/MEK1 in visfatin-induced signal transduction in Me45 melanoma cells.

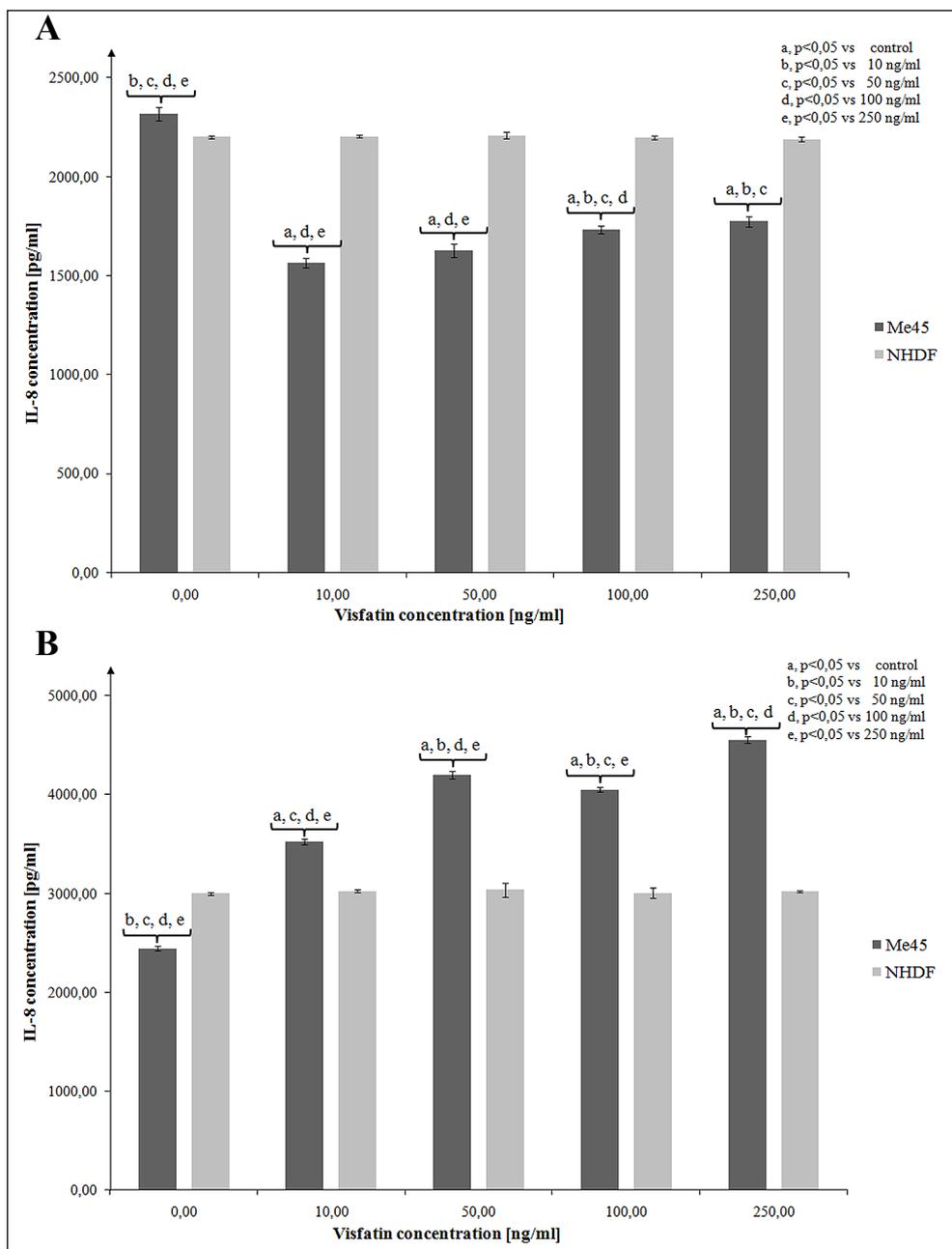


Fig. 2. IL-8 secretion in culture of melanoma Me45 cells and human fibroblasts after visfatin treatment 6 h (A) and 24 h (B) as determined by specific ELISA assay [pg/ml].

Additionally we performed experiments that showed effects of visfatin and protein kinases on IL-6 and IL-8 secretion in the presence of a specific NF- κ B inhibitor. We showed that addition of NF- κ B inhibitor prevented the rise in IL-6 concentration caused by visfatin (18.54 ± 0.92 pg/ml versus 31.49 ± 0.63 pg/ml). The co-administration of both kinase inhibitor and NF- κ B inhibitor to Me45 melanoma cells after visfatin treatment showed a minute but statistically significant decrease in IL-6 concentration when compared to cells treated with visfatin and PI3k inhibitor (17.21 ± 0.67 pg/ml versus 21.53 ± 0.74 pg/ml, $p < 0.05$); that may reflect other mechanisms involved in the activation of NF- κ B by visfatin (Fig. 3A). Similar effects were seen in the experiments exploring the IL-8 secretion (Fig. 3B).

As shown in Fig. 4, visfatin significantly increased active DNA binding p65 (Rel A) during first 10 hours of incubation reaching a peak 24 hours after visfatin stimulation. Between 24 and 48 hour of the incubation, the level of p65 activity (Rel A) was decreased in this study group. However, DNA-binding activity of p65 (Rel A) was higher in visfatin treated cells when

compared to untreated cells. In control Me45 cells, DNA-binding activity of p65 (Rel A) did not change in broad range. NF- κ B activation may also be dependent on ROS production in Me45 melanoma cells upon visfatin treatment. As shown in Fig. 5, level of intracellular ROS in Me45 melanoma cells treated with visfatin (100 ng/ml) for 24 hours was elevated in comparison to untreated cells; (595 RFU ± 9.1 versus 176 RFU ± 4.64).

DISCUSSION

Recent data have demonstrated that visfatin induces pro-inflammatory responses through activation of NF- κ B (27, 28) and that NF- κ B can further mediate reactive oxygen species production through the synthesis of pro-inflammatory cytokines. In our study we observed an increase in the secretion of pro-inflammatory cytokines (IL-6 and IL-8) in Me45 melanoma cells upon visfatin stimulation. These effects of visfatin can be

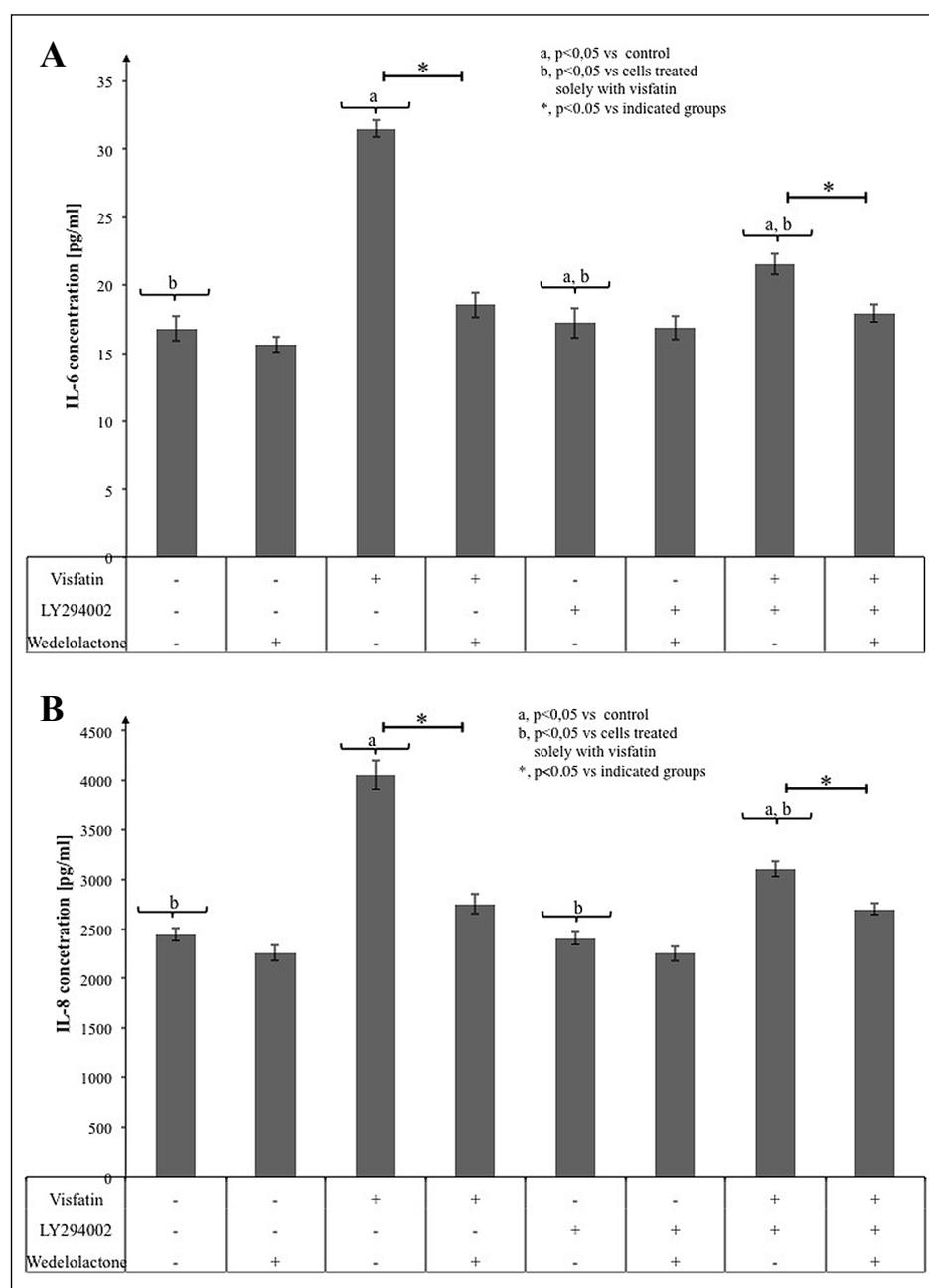


Fig. 3. PI3k inhibitor and NF- κ B antagonist abrogate visfatin-induced IL-6 and IL-8 cytokine secretion in Me45 melanoma cells. Human melanoma Me45 cells were treated with 100 ng/ml visfatin in the presence or lack of specific pharmacological kinase inhibitor for PI3k (LY204002) and wedelolactone for 24 hours. Concentration of IL-6 (A) and IL-8 (B) cytokine in supernatants after 24 hours were determined by ELISA assay. ^a $p < 0.05$ versus control (untreated cells), ^b $p < 0.05$ versus cells treated solely with visfatin, * $p < 0.05$ versus indicated groups.

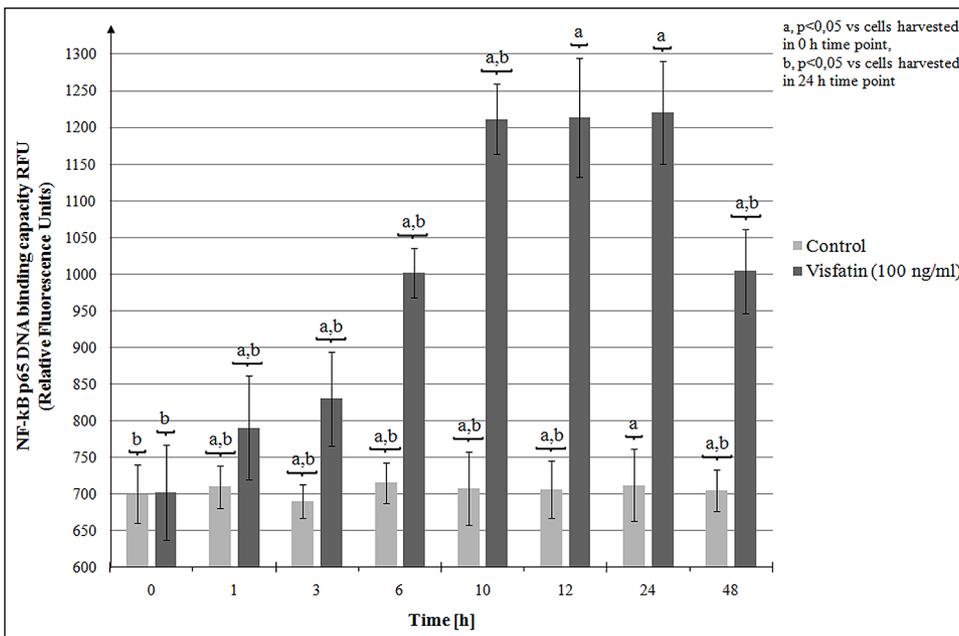


Fig. 4. Visfatin increases NF-κB p65 (RelA) DNA binding capacity in human malignant melanoma Me45 cells. Me45 cells were incubated with visfatin (100 ng/ml) and p65 binding capacity was determined by chemiluminescent transcription factor assay at the indicated time points (0–48 h). Data are expressed as relative fluorescence units (RFU), (n=6), (^a p<0.05 versus cells harvested at 0 h time point, ^b p<0.05 versus cells harvested at 24 h time point).

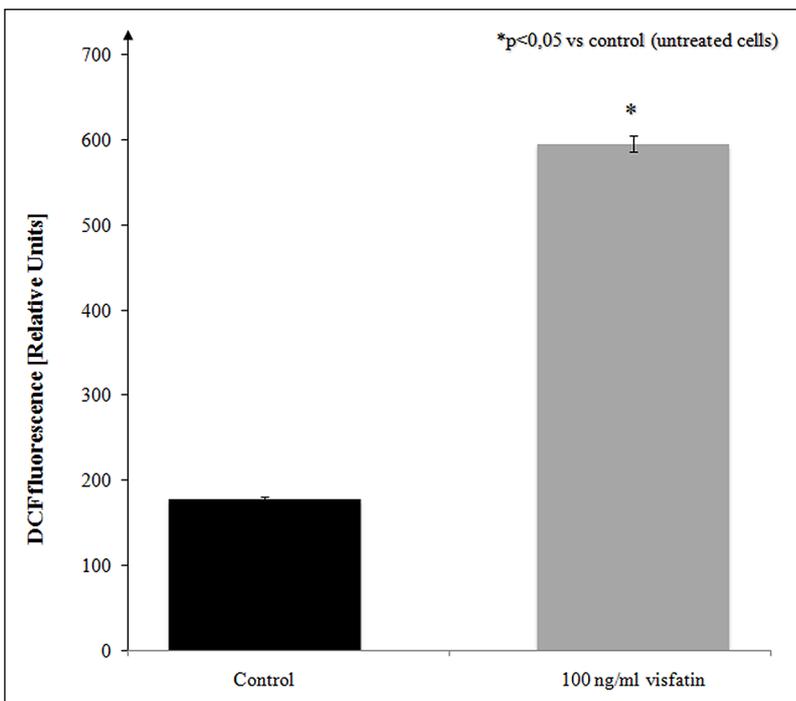


Fig. 5. The effect of visfatin on ROS production in Me45 melanoma cells after 24 h time of incubation. DCF-detectable ROS were measured in control (untreated cells), and visfatin-exposed cells (24 h exposure). Values were expressed as relative fluorescence units (RFU). Data are mean ± S.D. (n = 7). *, p<0.05 versus control untreated cells.

attributed to the similar signal transduction pathway observed in Romacho *et al.* (27) and Lee *et al.* (28) studies. It has been reported that visfatin also augmented oxidative stress *via* increased level of ROS in differentiated myotubes (29) and endothelial cells (30). Our previous study demonstrated an increase in the antioxidant enzymes activity and proliferation rate in those cells due to the increase in ROS level upon visfatin treatment of Me45 melanoma cells (24).

In our present study, all tested concentrations of visfatin led to a significant increase in IL-6 and IL-8 level in the supernatants of Me45 cells. However we did not observe significant differences between both cytokine secretion (IL-6, IL-8) in human normal human dermal fibroblasts treated with visfatin when compared to un-treated cells. In the study of Li *et al.* (31), visfatin was also a potent inducer of IL-6 secretion

and STAT3 tyrosine phosphorylation in cholesterol-laden macrophages *in vitro*. This effect was seen with a visfatin concentration as low as 50 ng per ml. They also showed that visfatin potently blocked macrophages apoptosis induced by a number of ER stressors (31). Recently Moschen *et al.* (18), reported pro-inflammatory properties exerted by visfatin by showing that it dose dependently up-regulated the production of the pro-inflammatory cytokines: IL-1β, IL-6, and TNF-α in human monocytes. Furthermore, IL-6 secretion by melanoma cells has not been previously studied in relation to visfatin stimulation. IL-6 effects on melanoma cells appear to be variable, depending on the tumor cell lines and on the stage of the disease (32). In fact, antisense oligonucleotides blocking IL-6 gene expression inhibited the growth of melanoma cell lines, suggesting that IL-6 promotes advanced stage melanoma

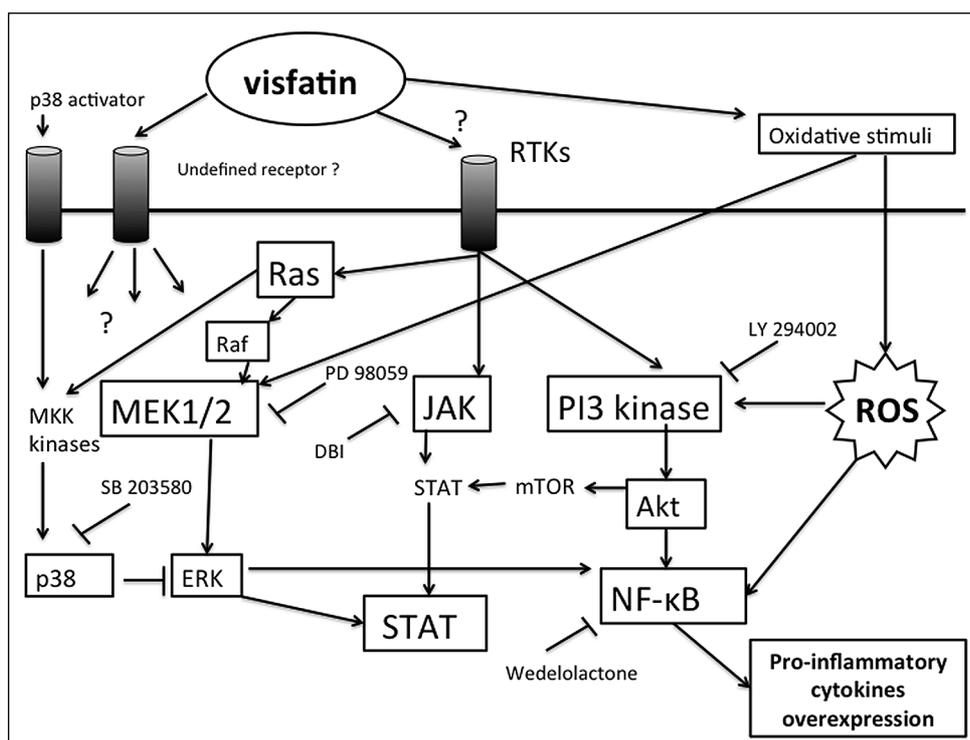


Fig. 6. Kinases signaling pathways and reactive oxygen species (ROS) involvement in the transduction of pro-inflammatory and oxidative stimuli. Specific inhibitors of p38, Janus kinase (JAK), MEK1, phosphatidylinositol 3'-kinase (PI3k) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) that were implemented in the study have been marked on the figure. Akt, also known as protein kinase B (PKB); ERK, extracellular signal-regulated kinases; MKK, mitogen-activated protein kinase; RTK, receptor tyrosine kinases; STAT, Signal transducer and activator of transcription.

cell growth by an autocrine mechanism (33). Moreover, melanoma Me45 cells showed a pattern of cytokine production different from that of fibroblasts. Among the malignant cells, we detected low amount of IL-6 in culture supernatants, while the non-malignant fibroblasts produce up to 100-fold more IL-6 than melanoma Me45 cells. IL-6 is produced by many different cell types, but fibroblasts are one of the important sources of this cytokine *in vivo* (34). Nevertheless, visfatin stimulation led to a significant increase in IL-6 secretion compared to the untreated melanoma Me45 cells which may stem from mild reactive oxygen species increase as well as NF-κB activation.

We also observed higher secretion of IL-8 by human malignant melanoma Me45 cells after stimulation with visfatin at the concentration of 250 ng per ml for 24 hours compared to A375M malignant (highly metastatic) melanoma cells in the similar culture conditions studied by Varney *et al.* (35), (4553 pg/ml versus 2300 pg/ml; respectively). Moreover, basal IL-8 level in culture supernatants of Me45 melanoma cells without adding visfatin was comparable to that obtained in the culture supernatants of A375M melanoma cells studied by those authors in the similar culture conditions (35). In another study, Enzmann and co-workers detected the highest amount of IL-8 in supernatants of choroidal melanomas, while the low metastatic Mel-Juso melanoma cells produced up to 2-fold less IL-8 than highly metastatic melanoma cells (36). Our data suggest that visfatin after 24 hours of incubation enhanced the secretion of IL-8 by malignant melanoma Me45 cells. We conclude that visfatin is a potent stimulator of IL-8 secretion in these cells. IL-8 has been implicated in melanoma progression through several mechanisms, including the promotion of chemotaxis, tumor growth and migration (37, 38). The role of IL-8 in melanoma progression has previously been primarily attributed to its ability to act as an autocrine growth factor for melanoma cells (38, 39) and to induce haptotactic migration (35). Taken together with the growth-promoting capacity of IL-6, IL-8 regarding human melanoma cells as demonstrated in other studies IL-6 and IL-8 might be an important factor that induces melanoma progression, proliferation and possibly selection of metastatic

tumor clones (35, 40, 41). In our previous work, visfatin at the same culture conditions of Me45 melanoma cells led to increase of proliferation rate, probably due to increase in level of growth promoting cytokines: IL-6, IL-8 in a NF-κB dependent manner as demonstrated in present study. Me45 melanoma cells were also stimulated with 100 ng/ml visfatin and specific kinase inhibitors were used to achieve insight into possible mechanisms of signaling pathway. Activation of NF-κB transcription factors is a central event in the initiation and amplification of inflammatory responses (42). We therefore analyzed activation of p65 (RelA), part of the p50:RelA dimer that is activated by the classical pathway in melanoma cells. NF-κB plays a central role in triggering and coordinating immune reactions including upregulation of cytokines such as: IL-6 (43). Moschen *et al.* (18) also observed that visfatin upregulated NF-κB p65 (RelA) DNA binding capacity in human leukocytes. In our present study, PI3k inhibitor also repressed visfatin-induced IL-6, IL-8 secretion. Despite the fact that we did not measure the direct effect of PI3k activation we speculate that this pathway is involved in the visfatin signalling in the Me45 cells. We did not observe similar effects when the specific inhibitors of MAPK/p38 and JAK/STAT pathways were used. Nevertheless it must be kept in mind that the other mechanisms or kinases may affect the observed result. Because PI3k is involved in the amplification of cell death pathway by activating the pro-survival kinase Akt (44, 45) activation of PI3k could be a possible mechanism involved in anti-apoptotic actions of visfatin as demonstrated previously published data (46, 47). According to experiments with NF-κB inhibitor we showed that the majority of effects exerted by PI3k is expressed through enhanced NF-κB pathway activity.

Finally, we speculated that visfatin led to the increase of cytokine production by increased intracellular reactive oxygen species level. ROS itself can activate NF-κB signalling pathway to increase of cytokine production in these cells. Taken together we conjecture that visfatin elevates interleukins partly by stimulation of PI3k pathway but also it is possible that part of the effects is a resultant of increased oxidative stress caused by visfatin. Kinase signaling pathways and reactive oxygen species involvement have been shown in the *Fig. 6*.

Limitation of the study should always be kept in mind. The *in vitro* setting may not reflect the myriad interactions occurring *in vivo*. Nevertheless the study clearly shows that visfatin exerts its potentially pathogenic effects on Me45 melanoma cells *via* PI3k kinase. This observation may be an important factor in the incorporation of the new therapeutic approach in the treatment melanoma (48), similarly to other malignant diseases (*e.g.* Non-Hodgkin lymphoma) (49). We also did not perform experiments on the invasive or metastatic potential of Me45 stimulated with visfatin (*e.g.* transwell matrigel invasion assay, scratch/wound healing assay). This issue needs further studies.

The supraphysiological concentration of visfatin in culture medium was similar to the concentrations used in other *in vitro* studies (16, 19, 23, 24, 29, 50, 51).

Abbreviations: JAK/STAT pathway, Janus kinase and signal transducer and activator of transcription pathway; MAPK/Erk pathway, mitogen-activated protein kinases, originally called extracellular signal-regulated kinases pathway; MKK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin kinase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells, NHDF, normal human dermal fibroblasts; PCOS, polycystic ovary syndrome; PI3k/Akt kinases, phosphatidylinositol 3'-kinase /Akt, also known as protein kinase B; PKB, protein kinase B; ROS, reactive oxygen species; RTKs, receptor tyrosine kinases;

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