Visfatin is actively secreted *in vitro* from U-937 macrophages, but only passively released from 3T3-L1 adipocytes and HepG2 hepatocytes

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Short title:

Secretion of visfatin from cell lines

Abstract

Visfatin is a multi-functional molecule that can act intracellularly and extracellularly as an adipokine, cytokine and enzyme. One of the main questions concerning visfatin is the mechanism of its secretion; whether, how and from which cells visfatin is released. The objective of this *in vitro* study was to observe the active secretion of visfatin from 3T3-L1 preadipocytes and adipocytes, HepG2 hepatocytes, U-937, THP-1 and HL-60 monocytes and macrophages. The amount of visfatin in media and cell lysate was always related to the intracellular enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to exclude the passive release of visfatin. Visfatin was not found in media of 3T3-L1 preadipocytes. In media of 3T3-L1 adipocytes and HepG2 hepatocytes, the ratio of visfatin to the amount of GAPDH was identical to cell lysates. Hence, it is likely that these cells do not actively secrete visfatin in a significant manner. However, we found that significant producers of visfatin are differentiated macrophages and that the amount of secreted visfatin depends on used cell line and it is affected by the mode of differentiation. Results show that 3T3-L1 adipocytes and HepG2 hepatocytes released visfatin only passively during the cell death. U-937 macrophages secrete visfatin in the greatest level from all of the tested cell lines

Abbreviations

pre-B cell colony-enhancing factor (PBEF), nicotinamide adenine dinucleotide (NAD), nicotinamide phosphoribosyltransferase (Nampt), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Minimum essential medium (MEM), Roswell Park Memorial Institute 1640 (RPMI-1640), phorbol 12-myristate 13-acetate (PMA), serum-free medium (SFM), trichloroacetic acid (TCA),

sodium dodecyl sulfate (SDS), polyacrylamide gel electrophoresis (PAGE), white adipose tissue (WAT)

Text

Visfatin was discovered as an insulin-mimetic adipokine produced predominantly by visceral adipose tissue (Fukuhara *et al.* 2005). This multi-functional molecule exhibits intracellular and also extracellular action. Besides its function as an insulin-mimetic adipokine, visfatin is also effective as an inflammatory cytokine pre-B cell colony-enhancing factor (PBEF) and as an important enzyme catalysing the rate limiting step in the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway – nicotinamide phosphoribosyltransferase (Nampt) (Rongvaux *et al.* 2002, Sommer *et al.* 2008). This enzymatic reaction, as well as visfatin function, is located both intracellularly (Kitani *et al.* 2003, Tanaka *et al.* 2007) and extracellularly (Jacques *et al.* 2012, Zhao *et al.* 2014). There is still a lot of controversy about this protein. The first issue is the function of visfatin as a secreted cytokine and adipokine. Some authors associate its extracellular function with the production of NAD (Revollo *et al.* 2007), because the membrane receptor of visfatin on target cells has thus far not been conclusively determined. Conversely, other authors describe the action of visfatin independently on its enzyme activity and via association with signalling kinase cascade (Garten *et al.* 2015, Lin *et al.* 2016, Soncini *et al.* 2014). Another issue of visfatin represents its mechanism of secretion. Even though its extracellular function has been described, the process of how visfatin is released from cells is still unclear.

Visfatin is not secreted by a classical secretory pathway using endoplasmic reticulum and Golgi complex and it has been hypothesized that visfatin is actively secreted by an unknown non-classical pathway (Tanaka *et al.* 2007). According to the visfatin function, adipocytes and

immune cells are described as the most important producers of this protein to the extracellular space (Curat *et al.* 2006, Garcia-Serrano *et al.* 2015, Tanaka *et al.* 2007). However, some ability to release visfatin has been detected in many cell types, including hepatocytes (Garten *et al.* 2010), glia cells (Zhao *et al.* 2014), pancreatic β-cells (Kover *et al.* 2013), melanoma cells (Grolla *et al.* 2015) and endothelial cells (Romacho *et al.* 2013). Nevertheless, the secretory mechanism and cellular receptor of visfatin have not been determined yet and thus the question about its active secretion remains unresolved. The presence of visfatin in the extracellular space might be due to its non-specific release during cell death and lysis, at least in certain case of cell types (Kitani *et al.* 2003, Stephens and Vidal-Puig 2006, Zhao *et al.* 2014).

In this short communication report, we examined the active secretion of visfatin from different cell types *in vitro* – mouse 3T3-L1 preadipocytes and adipocytes; human HepG2 hepatocytes; and human U-937, HL-60 and THP-1 monocytes and macrophages. To exclude passive release during cell death and lysis, internal control of active secretion must be performed. Simultaneously with the target protein, it is necessary to quantify the non-secreted intracellular protein control in culture media and cell lysates. The value that reflects active secretion is the increased ratio of quantities of the target protein and non-secreted protein control in media compared to cell lysates. Selection of the protein control is a critical step: It should be located in the same compartment as studied protein and must not be able to form multimeric structures or associate with membranes, because these factors affect its release during cell death and lysis. For instance, β-actin which is frequently used, is not one of the most eligible candidate for polymerisation. In this study, we used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the intracellular protein control. GAPDH is a soluble cytosolic enzyme; therefore, its passive

release during cell death should be the same as visfatin.

3T3-L1 preadipocytes were maintained in DMEM (HyClone) containing 10% FBS (Biochrom) and 4 $mmol.l^{-1}$ L-glutamine (HyClone). They were subsequently differentiated into adipocytes, as was described previously (Skop *et al.* 2014), using dexamethasone, isobutylmethylxanthine and insulin. HepG2 hepatocytes were maintained in MEM (HyClone) supplemented with 10% FBS, 2 $mmol.l^{-1}$ L-glutamine and non-essential amino acids (Sigma). U-937, HL-60 and THP-1 monocytes were maintained in RPMI-1640 medium (HyClone) containing 10% FBS and 2 $mmol.l^{-1}$ L-glutamine. They were subsequently differentiated into macrophages by incubation with 100 $\mu g.l^{-1}$ of phorbol 12-myristate 13-acetate (PMA, Sigma) for 24 h (U-937: 100 $\mu g.l^{-1}$, HL-60 and THP-1: 200 $\mu g.l^{-1}$). During this time, most of cells adhered to the dish surface. Next, the medium was exchanged for a medium without PMA, while all PMA-treated non-adherent cells were centrifuged and returned to the original dish to finish differentiation. Cells were fully differentiated after 48 h in the absence of PMA. To analyse the content of visfatin and GAPDH in lysates and media, cells were washed and cultivated in a serum-free medium (SFM) for 18 h. Thereafter, the medium was collected and centrifuged (200 rcf, 5 min). Proteins from supernatants were precipitated using trichloroacetic acid (TCA) in a final concentration of 0.575 $mol.l^{-1}$ (1 h on ice). Precipitated proteins were sedimented by centrifugation (18 000 rcf, 20 min, 2 °C). The pellet was resuspended in a loading buffer (50% glycerol, 2.5 $mol.l^{-1}$ Tris-HCl, pH 6.8, 25% 2-mercaptoethanol, 0.35 $mol.l^{-1}$ SDS, 0.5% bromophenol blue) and the rest of the TCA was neutralised by NaOH. This sample was run in SDS-PAGE. After cultivation in SFM, the cells were lysed using a lysis buffer (0.01 $mol.l^{-1}$ NaH_2PO_4, 0.01 $mol.l^{-1}$ Na_2HPO_4, 0.15 $mol.l^{-1}$ NaCl, 1% sodium deoxycholate, 1% Triton X-100) and sonication. Forty µg of the total proteins (measured using BCA assay, Pierce) were run in SDS-PAGE. After

electrophoresis, proteins were electro-transferred to nitrocellulose membranes, which were then blocked by 3% bovine serum albumin (Sigma). Blots were incubated with a mixture of primary rabbit antibodies directed against visfatin (1:5 000, RB-08-0003, RayBiotech) and GAPDH (1:20 000, G9545, Sigma). Antibody binding was then revealed with peroxidase-labelled mouse secondary antibody directed against rabbit IgG (1:5 000, W401B, Promega). Membranes were visualised using chemiluminescent reagents (West Pico, Pierce). Quantification of the bands was performed using Image J software (NIH, USA). One-way ANOVA followed by Tukey's test ($p < 0.05$) was performed to compare media against lysate.

The results dealing with secretion of visfatin from 3T3-L1, HepG2 and U-937 cells are shown in Fig. 1. When studying secretion of visfatin from 3T3-L1 cells, we found neither visfatin nor GAPDH in culture media of preadipocytes. Differentiated 3T3-L1 adipocytes always released visfatin with GAPDH and the ratio of the content of these two proteins in media was approximately equal to the ratio of intracellular content. In HepG2 hepatocytes, we obtained a similar result to 3T3-L1 adipocytes, which suggests that visfatin is released from these two cell lines during spontaneous cell lysis. A different situation occurred in the case of U-937 cells. U-937 monocytes exhibited a mildly increased visfatin-to-GAPDH ratio in media compared to lysates, indicating a low level of active visfatin secretion. Interestingly, differentiated U-937 macrophages released a high amount of visfatin in media, while GAPDH was only weakly detected there. In addition, the ratio of the content of visfatin-to-GAPDH was substantially higher in media compared to cell lysates, demonstrating the active secretion of visfatin from this cell line.

Two another human monocyte lines with macrophage differentiation ability (THP-1 and HL-60) were tested on active secretion of visfatin to confirm the tissue specificity of macrophages as

cells actively secreting visfatin. Furthermore, various differentiation protocols of U-937 cells were used to study whether mode of differentiation affects visfatin secretion. THP-1 and HL-60 macrophages exhibited significantly increased visfatin-to-GAPDH ratio in media compared to lysates, which shows that both of these cell lines actively secreted visfatin (Fig. 2A). Results further show that from tested macrophage cell lines, U-937 cells secreted the greatest level of visfatin. Moreover, the quantity of visfatin secreted into media depends on the differentiation protocol in U-937 cell line (Fig. 2B), where the amount of secreted visfatin increases with increasing concentration of the differentiation inducer, PMA.

The question concerning the mechanism of visfatin secretion remains unanswered. Our results show that 3T3-L1 preadipocytes, adipocytes and HepG2 hepatocytes do not secrete visfatin actively. However, this is not in agreement with some previously reported studies (Garten *et al.* 2010, Tanaka *et al.* 2007). Secretion of visfatin from 3T3-L1 adipocytes was reported for the first time by Fukuhara *et al.* 2005 in the original article describing visfatin as an adipokine. However, an internal control of active secretion was absent from this study. Moreover, the retraction of the article two years after publication caused further doubt on the validity of their results (Fukuhara *et al.* 2007). The same scientific group published an article dealing directly with secretion of visfatin from 3T3-L1 cells (Tanaka *et al.* 2007), however, no comparison with a non-secreted protein control or any assay of cell death or lysis was performed. In other reports that studied the influence of effectors on the secretion of visfatin from 3T3-L1 cells, there has been an absence of any internal control of active secretion and visfatin concentration in media was very low (Eseberri *et al.* 2013, Li *et al.* 2014).

Thus far, only one work has examined the secretion of visfatin from HepG2 hepatocytes (Garten *et al.* 2010). In this study, the active secretion of visfatin was found from HepG2 cells and

primary hepatocytes. To exclude passive release, the level of cell death was determined by adenylate kinase assay. Although there was no increase in cell death parameters over a 3-day cultivation in serum-free media, the concentration of visfatin in media increased continually during that time (Garten *et al.* 2010). On the contrary, in our experiments, we were not able to cultivate HepG2 cells in serum-free media without a certain level of cell death. This was accompanied by the release of GAPDH and visfatin into the extracellular space. Due to this problem, we cannot exclude the active secretion of visfatin in some small extent by HepG2 cells, because, theoretically, the low concentration of actively secreted visfatin would be obscured by the higher level of visfatin released during cell death. According to our results, HepG2 hepatocytes and 3T3-L1 adipocytes do not seem to produce visfatin in significant quantities and, therefore, these cell lines do not provide a good model for studying visfatin secretion.

Because secretion of visfatin from macrophages has been previously shown (Curat *et al.* 2006, Schilling and Hauschildt 2012, Wang *et al.* 2016), the other cell types we examined were U-937, THP-1 and HL-60 monocytes and macrophages. All of tested human macrophages differentiated by PMA substantially secreted visfatin. We thus hypothesise that macrophages are the main producers of visfatin. This hypothesis is in agreement with results showing increased serum levels of visfatin in obesity and metabolic syndrome (Chang *et al.* 2011), as macrophages naturally occurs in white adipose tissue (WAT) and their number and production of cytokines is increased in obesity (Ghazarian *et al.* 2015). Since the amount of secreted visfatin differs between tested macrophage cell lines, we further tested whether mode of differentiation affects visfatin secretion. We found that the amount of visfatin secreted from U-937 macrophages was significantly affected by concentration of the differentiation agent PMA, even though all of these cells had a macrophage phenotype (adherent growth, stopped cell division) and intracellular

visfatin content only slightly differed. Various surrounding conditions during macrophage differentiation and activation in body result in the formation of diverse macrophage sub-populations, which secrete diverse spectra of cytokines (Mcnelis and Olefsky 2014, Murray and Wynn 2011). We may thus suppose that visfatin is secreted from diverse macrophage sub-populations in a different manner.

We may conclude that, in our *in vitro* experiments, U-937, THP-1 and HL-60 macrophages significantly and actively secreted visfatin, but in case of 3T3-L1 adipocytes or HepG2 hepatocytes active secretion of visfatin was not observed. The highest secretion of visfatin was found in U-937 macrophages, therefore, this cell line is the most suitable for visfatin secretion mechanism study from all of the tested cells. Since visfatin serum content has been hypothesised to play an important role in type 2 diabetes or cardiovascular disease (Sun *et al.* 2013), our results provide a new insight into the complex role of macrophages in the pathogenesis of such diseases.

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Figures

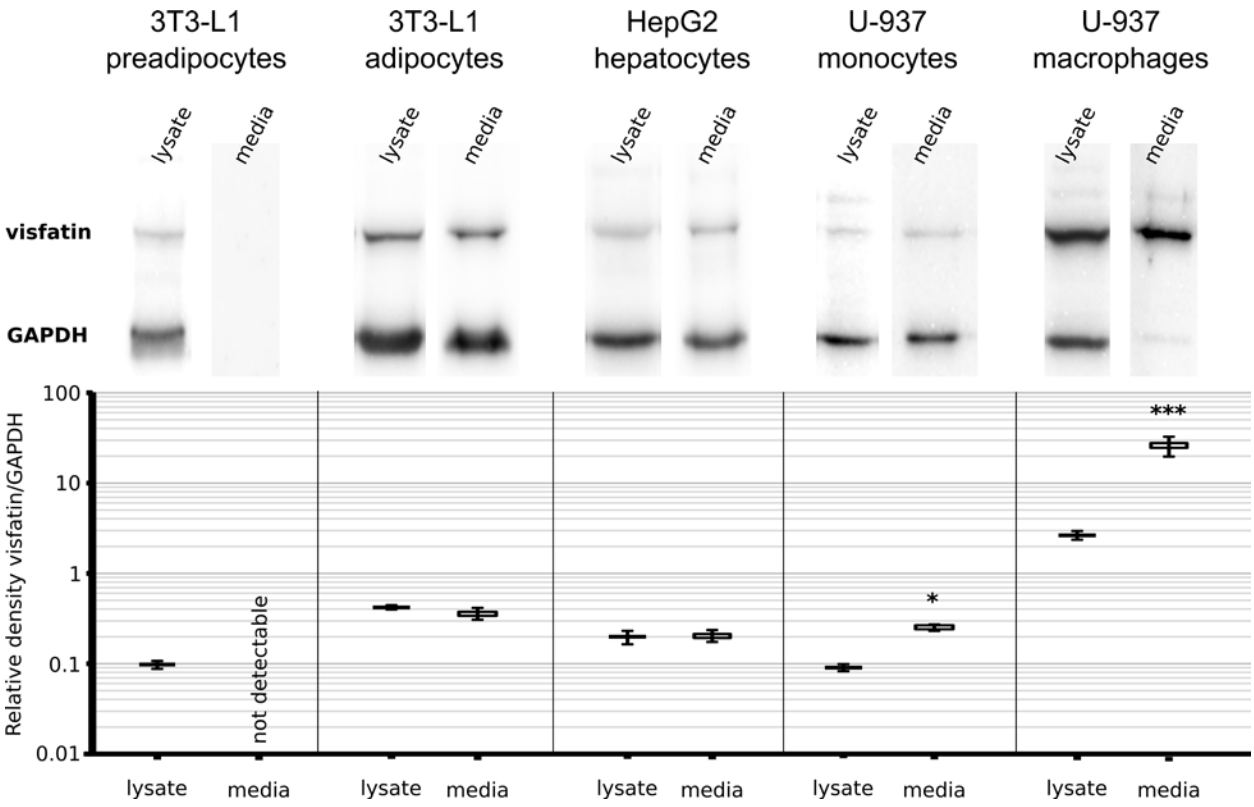

Fig. 1. Representative Western blots and quantification of intracellular and extracellular visfatin

and GAPDH from 3T3-L1 preadipocytes, 3T3-L1 adipocytes, HepG2 hepatocytes, U-937 monocytes and U-937 macrophages. Values are mean ± SE, n=3. ANOVA analysis: * P<0.05, *** P<0.01 media vs. lysates.

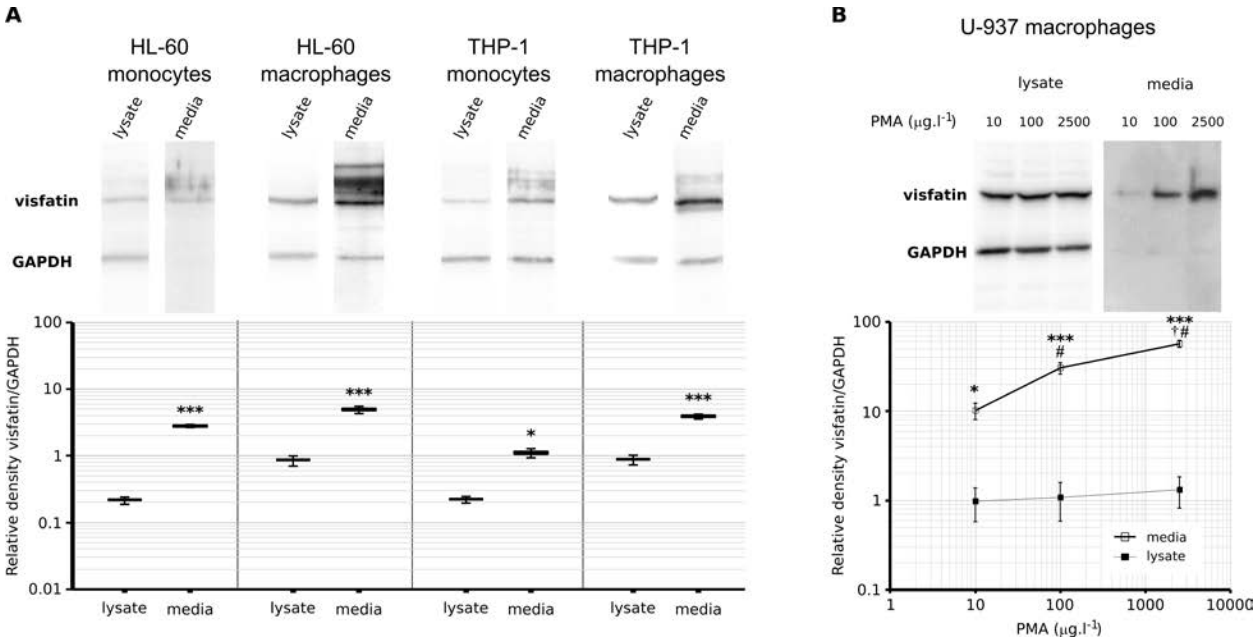

Fig. 2. Representative Western blots and quantification of intracellular and extracellular visfatin and GAPDH from HL-60 and THP-1 monocytes and macrophages (A), U-937 macrophages differentiated using various concentrations of PMA (B). Values are mean ± SE, n=3. ANOVA analysis: * P<0.05, ***P<0.01 media vs. lysates, #P<0.05 vs. 10 µg.l^{-1} PMA, †P<0.05 vs. 100 µg.l^{-1} PMA.