Visfatin/Nampt induces telomere damage and senescence in human endothelial cells

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ARTICLE INFO

Article history:
Received 23 April 2014
Accepted 12 May 2014
Available online 17 May 2014

Keywords:
Visfatin
Nampt
Endothelium
Senescence
DNA damage
Telomere

The adipose tissue is an active endocrine organ that releases a wide array of substances, globally termed adipokines. Among these, visfatin is a multifaceted molecule, structurally identical to the extracellular form of nicotinamide phosphoribosyltransferase (Nampt), which enzymatically converts nicotinamide into nicotinamide mononucleotide (NMN) and whose circulating levels are enhanced in metabolic disorders (reviewed in [1]).

Obesity, the metabolic syndrome and type 2 diabetes are being increasingly recognized as causative of premature vascular aging even though the underlying mechanisms are only starting to be elucidated [2,3]. Vascular aging, which is currently regarded as a cardiovascular risk factor, is characterized by a series of morphological and functional alterations including endothelial dysfunction, arterial stiffness, calcification and cell senescence [4]. Evidence that adipokines could play a role in the development of these underlying phenomena is limited. Here, we explored whether visfatin/Nampt plays a role in promoting premature human endothelial cell senescence. The study was approved by the ethics committee of Universidad Autónoma de Madrid and Hospital Universitario de Getafe.

Treatment of cultured human umbilical vein endothelial cells (HUVEC) with visfatin/Nampt increased the fraction of cells staining positive for senescence-associated-β-galactosidase (SA-β-gal), a marker of cell senescence [5], in a concentration-dependent manner, with 50 ng/ml being the lowest concentration tested that caused a significant effect (Fig. 1A and B). The pro-senescence effect of visfatin/Nampt (50 ng/ml) was time-dependent, and similar in its extent to that induced by exogenously added H_2O_2 (50 μmol/l), with over 60% of the cells becoming SA-β-gal$^+$ after 96 h exposure in both cases (data not shown). These results raised the possibility that visfatin/Nampt might induce senescence by causing oxidative stress. Indeed, visfatin/Nampt stimulated NADPH oxidase activity (Fig. 1C), a major source of reactive oxygen species in endothelial cells, as determined by lucigenin-derived chemiluminescence [6]. Furthermore, the NADPH oxidase inhibitor apocynin (30 μmol/l) prevented the increase in SA-β-gal$^+$ cells elicited by visfatin/Nampt (Fig. 1D). These results demonstrate that NADPH oxidase activation mediates the induction of human endothelial cell senescence by visfatin/Nampt.

Premature senescence often results from oxidative stress causing extensive DNA damage, particularly at telomeres [7]. Hence, to dissect further the mechanism involved in visfatin/Nampt-induced senescence, we looked for the appearance of phosphorylated histone H2AX (γH2AX) foci, which are formed at sites of DNA damage and dysfunctional telomeres [8]. Immunofluorescence microscopy revealed that visfatin/Nampt caused a concentration-dependent increase in γH2AX foci, which were found scattered over the total nuclear DNA as well as co-localizing with telomeric TRF-1 signals in the so called telomere dysfunction-induced foci (TIFs) [9] (Fig. 2A and B). This effect was qualitatively similar to that seen when cells were treated with etoposide (20 μmol/l), a topoisomerase inhibitor known to cause extensive DNA damage (Fig. 2A). Furthermore, Western blot analysis revealed that after treatment with visfatin/Nampt, HUVEC exhibited higher p53 levels (Fig. 2D), in line with the concept that stabilization of this protein is a key downstream signaling event mediating the response to DNA damage and telomere dysfunction [10].

Different actions of visfatin/Nampt in the cardiovascular system have been reported to rely on Nampt enzymatic activity [1]. In agreement with this premise FK866 (10 μmol/l), a highly specific Nampt inhibitor [11], blunted all the observed effects of visfatin/Nampt, namely the activation of NADPH oxidase (Fig. 1C), the increase in SA-β-gal$^+$ cells (Fig. 1E), the increase in γH2AX foci and in cells positive for TIFs (Fig. 2C), and the rise of p53 levels (Fig. 2D). The involvement of Nampt activity was further substantiated by the fact that the product of its enzymatic reaction, NMN (100 μmol/l), raised NADPH oxidase activity (Fig. 1C), increased the number of SA-β-gal$^+$ cells (Fig. 1E), promoted the formation of γH2AX foci and TIFs (Fig. 2C), and enhanced cellular p53 levels (Fig. 2D), all to an extent similar to that seen in cells treated with visfatin/Nampt.

In conclusion, this work demonstrates that visfatin/Nampt promotes premature endothelial cell senescence by a mechanism that relays on its Nampt activity and on the activation of NADPH oxidase, and that ultimately results in the induction of DNA damage, telomere dysfunction and p53 up-regulation. These findings suggest that through its pro-senescence properties, visfatin/Nampt may contribute to vascular aging and its associated pathologies in the context of metabolic diseases.

This work was supported by Plan Nacional de I+D (SAF2011-28011, SAF2011-24648) and Fundación Eugenio Rodríguez Pascual. AU is the recipient of a Cardiff Metropolitan University Vice-Chancellor's Doctoral Scholarship. LV is the recipient of a fellowship from CONACYT, Mexico. TR is the recipient of a Marie Curie Intra-European Fellowship (2012-IEF-328793 ADDIO). CP and CFSF are engaged in the COST Action BM1005 ENOG.

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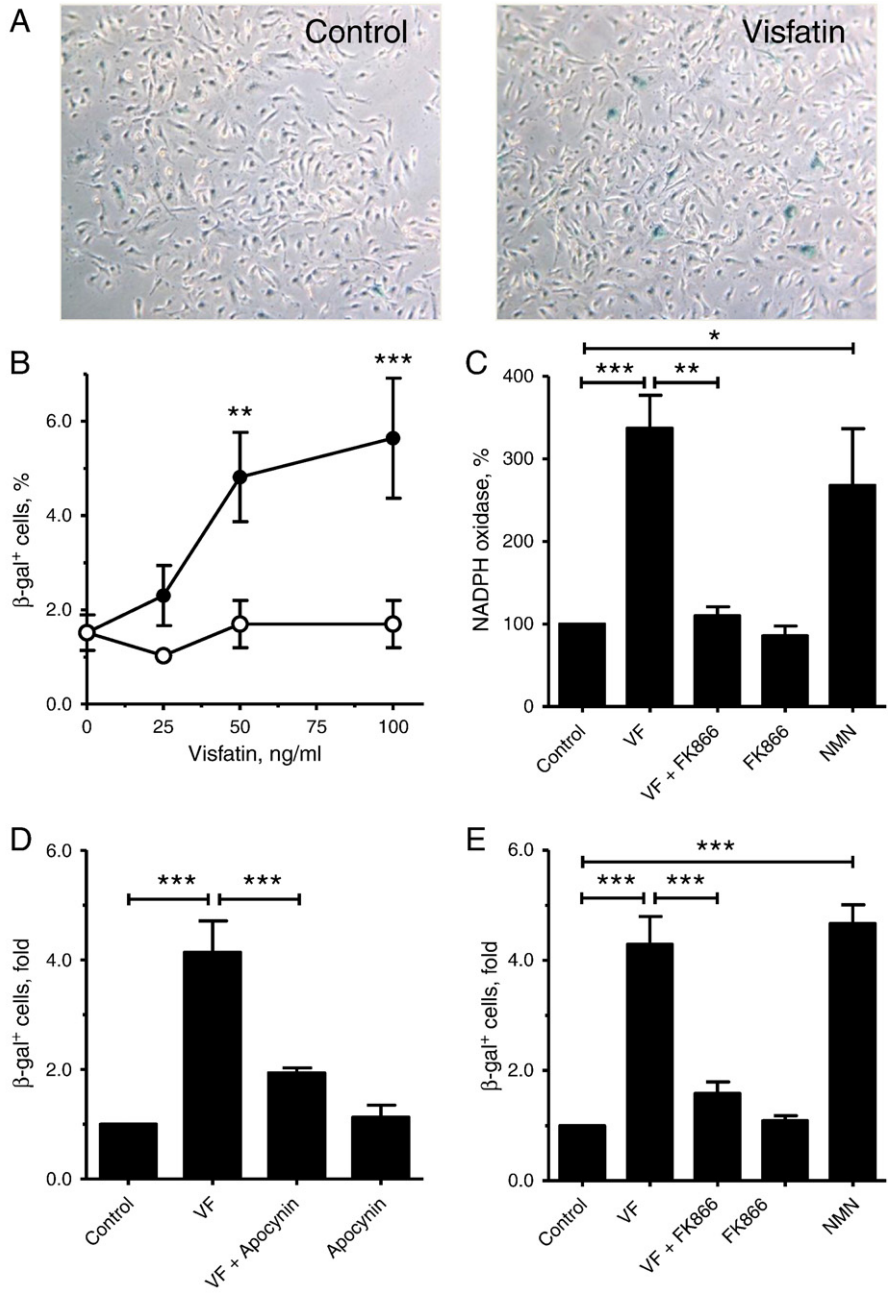

Fig. 1. Visfatin induces endothelial cell senescence via NADPH oxidase activation. HUVEC cultured as previously described [6] were treated for 24 h with the following compounds: 50 ng/ml visfatin (VF), 10 μmol/l FK866, 100 μmol/l NMN, and/or 30 μmol/l apocynin, or with various concentrations of VF as indicated. Senescent cells were detected by SA-β-gal staining [5]. The fraction of SA-β-gal positive (blue) cells was determined by manual scoring at least 800 cells in each sample under an inverted microscope. NADPH oxidase activity was measured in cell lysates by lucigenin-derived chemiluminescence [6]. (A) Phase contrast photomicrographs showing SA-β-gal staining and (B) Concentration-response for the effect of visfatin (filled symbols) vs vehicle (empty symbols) on the accumulation of SA-β-gal positive cells. Results are expressed as a percentage of the total number of cells in the fields of view; n = 5–7. (C) NADPH oxidase activity (n = 4) and (D, E) SA-β gal positive cells after the indicated treatments (n = 3–7). Results (mean ± SEM) are expressed relative to the values measured in untreated cultures; *n* denotes the number of independent experiments performed. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by one- or two-way analysis of variance (ANOVA) as appropriate, followed by Bonferroni's post hoc tests.

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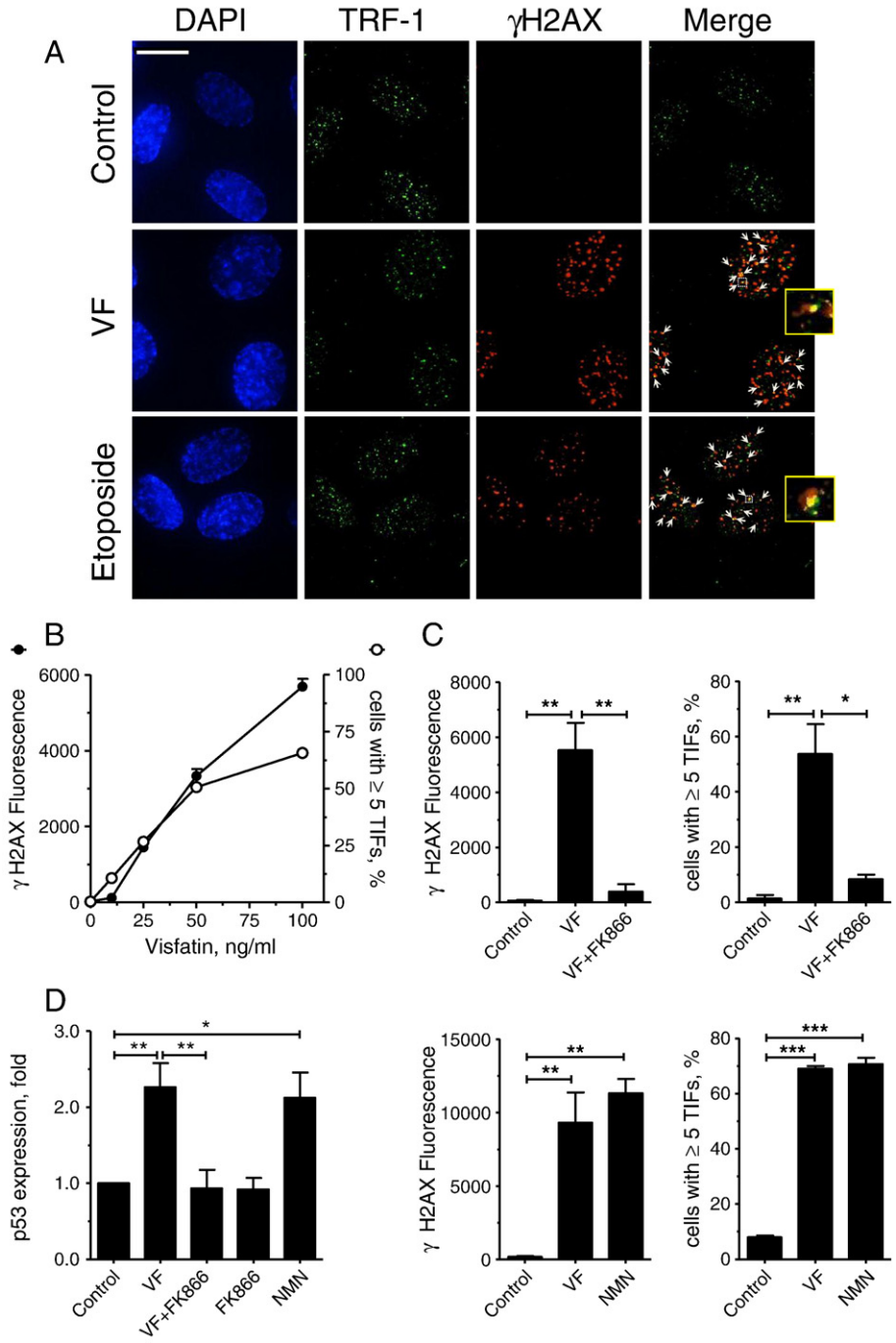

Fig. 2. Visfatin promotes DNA damage and telomere dysfunction. (A–C) Cultures were treated as described in Fig. 1 and then DNA damage foci and telomere dysfunction induced foci (TIFs) were detected by immunofluorescence microscopy as previously described [8] using antibodies against γH2AX (red) and TRF-1 (green). Cells were counterstained with DAPI (blue). (A) Representative deconvolved images of HUVEC treated with either 50 ng/ml visfatin or 20 μmol/l etoposide. Arrows in the merged images point to sites of colocalization. The rightmost small panels show enlarged views of the boxed areas in the merged images. Bar = 15 μm. (B) Dose–response for the effect of visfatin on the formation of γH2AX foci and TIFs expressed as mean fluorescent voxels/cell and as percentage of cells with ≥ 5 TIFs/cell, respectively and (C) quantification of γH2AX foci and TIFs after the indicated treatments. Data represent the average of 3 experiments with >200 cells scored in each case. (D) Western blot analysis of p53 protein expression 18 after treatment, calculated as the ratio of the intensity of the p53 band to the intensity of the corresponding actin bands. Results (mean ± SEM) are expressed relative to the values measured in untreated cells. n = 3–6. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by one way ANOVA, followed by Bonferroni's post hoc tests.

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http://dx.doi.org/10.1016/j.ijcard.2014.05.028