

## **Nuclear Factor- $\kappa$ B Induction by Visfatin in Human Vascular Endothelial Cells: Role in MMP-2/9 Production and Activation**

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**Running Title:** Visfatin induces NF- $\kappa$ B in Human Endothelial Cells

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## ABSTRACT

*Objective:* Visfatin is elevated in obesity and T2DM; and thought to be an inflammatory mediator within atherosclerotic lesions inducing gelatinase activity. We investigated the activation of NF- $\kappa$ B a well-known pro-inflammatory transcription factor by visfatin in ECs.

*Research Design and Methods:* Human ECs were transfected with pNF- $\kappa$ B-Luc plasmid. Using Quantitative PCR, western blot analysis and gelatin zymography, we studied NF- $\kappa$ B signalling in gelatinase mediated vascular inflammation by visfatin; employing the NF- $\kappa$ B inhibitor, BAY 11-7085.

*Results:* Visfatin significantly increased NF- $\kappa$ B transcriptional activity ( $P < 0.001$ ). Also, we found a significant inhibition of TNF $\alpha$  induced NF- $\kappa$ B activity by visfatin ( $P < 0.001$ ). Furthermore, the NF- $\kappa$ B inhibitor, significantly negated visfatin induced MMP-2/9 mRNA expression, protein levels, and gelatinolytic activity ( $P < 0.001$ ).

*Conclusions:* Visfatin induced NF- $\kappa$ B signalling in human ECs affects the activation of gelatinases-MMP-2/9, suggesting an important role of visfatin in the pathogenesis of vascular inflammation in obesity and T2DM.

## ABBREVIATIONS.

EC endothelial cell  
MMP matrix metalloproteinase  
NF- $\kappa$ B nuclear transcription factor kappa- $\beta$   
T2DM type 2 diabetes mellitus  
TNF- $\alpha$  tumour necrosis factor- $\alpha$   
VEGF vascular endothelial growth factor

Cardiovascular disease is more common in individuals with diabetes mellitus and obesity (1). Adipocytes and stromal vascular cells within adipose tissue directly augment systemic inflammation. Circulating mediators of inflammation participate in the mechanisms of vascular insult and atheromatous change, and many of these inflammatory proteins are secreted directly from adipocytes and adipose tissue-derived macrophages (2).

Visfatin, an adipokine, has been shown to be elevated in obesity, insulin resistance states and T2DM (3-5). More recently, it has been suggested that visfatin is an inflammatory mediator based on its localisation in macrophages within atherosclerotic lesions, and its ability to induce MMP-9 in monocytes (6). Moreover, we have described visfatin-inducing gelatinases (MMP-2/9) in human endothelial cells (7). Interestingly, systemic inflammation mediates multiple pathogenic mechanisms in the well-known associations between obesity, cardiovascular pathology, and co-morbidities such as T2DM and the metabolic syndrome (2); these associations, however, are poorly understood.

NF- $\kappa$ B is a major transcription factor in inflammatory responses, regulating a plethora of genes, playing a vital role in the initiation, progression and rupture of atherosclerotic plaques (8). Crucial enzymes involved in this process are the gelatinases (MMP-2 and MMP-9), the transcription of which is regulated by NF- $\kappa$ B (9)

With the aforementioned in mind, we sought to investigate whether visfatin activates NF- $\kappa$ B inducing inflammatory effects in the vascular endothelium.

## RESEARCH DESIGN AND METHODS

We studied NF- $\kappa$ B activation by visfatin, by stably transfecting a human endothelial cell line, EAHy926 (hybridoma of HUVECs and epithelioma A549 cells), or transient transfection of HUVECs, with a *cis*-reporter plasmid containing luciferase reporter gene linked to five repeats of NF- $\kappa$ B binding sites

(pNF- $\kappa$ B-Luc; Stratagene, La Jolla, CA). Multiple clones were selected for the analysis of NF- $\kappa$ B activation. Further, using Quantitative PCR, western blot analysis and gelatin zymography, we investigated the involvement of NF- $\kappa$ B signalling in gelatinase mediated vascular inflammation, by visfatin; employing the NF- $\kappa$ B inhibitor, BAY 11-7085 [see online appendix available at <http://care.diabetesjournals.org>].

## RESULTS

In pNF- $\kappa$ B-Luc stably transfected ECs, visfatin induced a significant dose dependent increase in NF- $\kappa$ B mediated transcriptional activity (Fig 1A); comparable potency to TNF- $\alpha$  (10ng/ml) [data not shown], a robust inducer of NF- $\kappa$ B activity. Similar significant results were obtained with transiently transfected HUVECs (on-line appendix). (Fig A).

Also, ECs preincubated with visfatin (dose-dependent) for 16 hours and then subjected to TNF $\alpha$  (10ng/ml) treatment for 2 hours revealed significant inhibition of TNF $\alpha$  induced NF- $\kappa$ B mediated transcriptional activity by visfatin (Fig 1B). Prior time-dependent experiments (0-24 hours) showed a maximal response at 2 hours (data not shown).

In light of our current observations that visfatin increases NF- $\kappa$ B transcriptional activity, we employed the NF- $\kappa$ B inhibitor, BAY 11-7085, to determine its role in visfatin mediated MMP activation. Interestingly, we found that visfatin induced MMP-2/9 mRNA expression and gelatinolytic activity were significantly negated with BAY 11-7085 (10 $\mu$ M) [Fig 1C-E].

Likewise in visfatin/ TNF- $\alpha$  treated ECs, MMP-2/9 protein levels were significantly decreased by pre-incubation with BAY 11-7085 (10 $\mu$ M) (on-line appendix) [Fig B1 & B2].

## CONCLUSIONS

We present novel data showing that visfatin is a profound stimulator of NF- $\kappa$ B

transcriptional activity in human endothelial cells. Also, our results demonstrate the crucial involvement of NF- $\kappa$ B signalling in visfatin induced activation of gelatinases; factors which are important in the pathogenesis of vascular inflammation.

Furthermore, we present novel data that visfatin induces hypo-responsiveness of NF- $\kappa$ B mediated transcriptional activity in human ECs. These findings are of importance given the fact that obesity and T2DM are states of pro-inflammatory cytokine ‘overload’ (10). It can be said, therefore, that this dysregulation of NF- $\kappa$ B signalling induced by visfatin in ECs may affect the fine balance in the variety of inflammatory responses present in these dysmetabolic states.

In vascular inflammatory responses, NF- $\kappa$ B signalling is an important regulator of endothelial adhesion molecules, chemokines, as well as MMPs (11); key enzymes involved in disruption of atherosclerotic plaques, and in vessel wall remodelling, as part of an inflammatory response. Interestingly, Dahl *et al* have recently suggested that visfatin may play a role in plaque destabilisation, given that macrophages are laden with visfatin, with the latter inducing MMP-9 in human THP-1 monocytes (6). More recently, we have demonstrated visfatin’s angiogenic potential in endothelial cells (7); dysregulated angiogenesis as seen in diabetes or chronic

inflammation involves the MMP system. Thus, activation of NF- $\kappa$ B by visfatin may play an important role in vascular pathology associated with obesity and T2DM. Our data supports this notion since visfatin induced MMP-2/9 production and activities were profoundly negated by the NF- $\kappa$ B inhibitor, BAY 11-7085.

The physiological/pathophysiological significance of our findings may pertain to the observation that visfatin levels are raised in obesity and diabetes, and that MMP-2/9 play critical role in vascular pathology. Given visfatin’s involvement in plaque destabilisation (6), our novel findings of NF- $\kappa$ B induction by visfatin in endothelial cells add a new perspective to visfatin’s proinflammatory role. The limitation of our *in vitro* study needs to be further clarified *in vivo*.

In summary, our findings introduce a novel insight into visfatin’s diverse roles in the development of the metabolic syndrome and reaffirm the emerging roles of adipokines as mediators of inflammatory responses.

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## FIGURE LEGEND

**Figure 1.** (A) Serum-starved ECs stably transfected with pNF $\kappa$ B-Luciferase were treated with or without visfatin (0-1600 ng/ml) for 2 hours. Cells were lysed and luciferase activities were measured. Visfatin induced a dose dependent increase in luciferase activity at 2 hours. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. basal, respectively (B) Serum-starved ECs stably transfected with pNF $\kappa$ B-Luciferase were pre-incubated with or without visfatin (0-1600 ng/ml) [16 hours] followed by TNF $\alpha$  (10ng/ml) for 2 hours. Similarly, cells were lysed and luciferase activities were measured. Results showed significant inhibition of TNF $\alpha$  induced NF- $\kappa$ B mediated transcriptional activity by visfatin. \*\*\* $P < 0.001$  vs. basal; ## $P < 0.01$ , ### $P < 0.001$  vs. TNF $\alpha$  treated ECs. (C) Serum starved ECs treated with visfatin (0-1600 ng/ml) for 4 hours showed a significant dose-dependent increase in MMP-2/9 : GAPDH mRNA expression, \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. basal, respectively. Furthermore, serum starved ECs treated with visfatin (1600 ng/ml) pre-incubated with BAY 11-7085 (10 $\mu$ M) for 1 hour significantly decreased MMP-2/9 mRNA expression. ### $P < 0.001$  vs. visfatin treated. (D-E) Serum starved ECs treated with visfatin (0-1600 ng/ml) for 24 hours showed a significant dose-dependent increase in MMP-2/9 gelatinolytic activity. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. basal, respectively. Furthermore, serum starved ECs treated with visfatin (1600 ng/ml) [24 hours] pre-incubated with BAY 11-7085 (10 $\mu$ M) for 1 hour significantly decreased MMP-2/9 gelatinolytic activity. ### $P < 0.001$  vs. visfatin treated. Data are means  $\pm$  SEM of three experiments. Each experiment was carried out triplicates.

FIGURE 1

