

Autocrine Effects of Visfatin on Hepatocyte Sensitivity to Insulin Action

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Summary

Visfatin was originally described as an adipokine with insulin mimetic effects. Recently, it was found that visfatin is identical with the *Nampt* (nicotinamide phosphoribosyltransferase) gene that codes for an intra- and extracellular NAD biosynthetic enzyme and is predominantly expressed outside adipose tissue. In the current study, we found strong protein and mRNA expression of visfatin in rat heart, liver, kidney, and muscle while the expression of visfatin in visceral fat was significantly lower and undetectable in subcutaneous fat. The insulin-mimetic effects of visfatin (extracellular form of *Nampt* or *eNampt*) are controversial and even less is known about autocrine effects of visfatin (intracellular form of *Nampt* or *iNampt*). Since liver plays the major role in glucose metabolism, we studied visfatin effects on insulin stimulated cellular glucose uptake in Fao rat hepatocytes using RNA interference (RNAi). RNAi mediated down regulation of visfatin expression in Fao cells was associated with significantly reduced NAD biosynthesis (0.3 ± 0.01 versus 0.5 ± 0.01 mmol/h/g, $P < 0.05$) and with significantly decreased incremental glucose uptake after stimulation with insulin when compared to controls with normal expression of visfatin (0.6 ± 0.2 versus 2.2 ± 0.5 nmol/g/2h, $P = 0.02$). These results provide evidence that visfatin exhibits important autocrine effects on sensitivity of liver cells to insulin action possibly through its effects on NAD biosynthesis.

Adipokines are bioactive mediators released from adipose tissue. The effects of adipokines on vascular function, immune system regulation and adipocyte metabolism make them key players in the pathogenesis of metabolic syndrome (Rajala *et al.* 2003). Recently, Fukuhara *et al.* (2005) reported identification of a new adipokine named visfatin (visceral fat adipokine). Several subsequent studies have focused on clarifying the expression, secretion and function of visfatin in glucose and lipid metabolism, but with conflicting results (Arner, 2006; Brendt *et al.* 2006; Chen *et al.* 2006; Sethi *et al.* 2005; Stephens *et al.* 2006). Later on, visfatin was found to be identical with the presumptive cytokine PBEF (pre-B cell colony-enhancing factor) (Samal *et al.* 1994) and finally with the *Nampt* (nicotinamide phosphoribosyltransferase) gene that is expressed predominantly outside the adipose tissue and that codes for an enzyme involved in nicotinamide adenine dinucleotide (NAD) biosynthesis (Rongvaux *et al.* 2002). Recently, Revollo *et al.* (2007) demonstrated that extracellular visfatin (*eNampt*) does not exert insulin-mimetic effects in vitro when exogenously administered visfatin showed no effects on differentiation of the human SGBS preadipocyte cell line or on glucose uptake in 3T3-L1 adipocytes. In their original paper, Fukuhara *et al.* (2005) reported insulin mimetic effects of visfatin also in liver cells when exogenous visfatin suppressed glucose release in H4IIEC3 hepatocytes. In the current study, we analyzed the expression of visfatin in rat tissues and studied visfatin effects on cellular glucose uptake and insulin sensitivity in Fao rat hepatocytes using RNA interference (RNAi) mediated down regulation of visfatin expression.

Figure 1A and 1B shows the expression of visfatin mRNA and protein in tissues isolated from Wistar Kyoto (WKY) rats detected by agarose gel electrophoresis of the PCR products through reverse transcription of the total RNA and by western blot analysis. As can be seen, no visfatin-specific PCR product was detected in the subcutaneous fat tissue. The

absence of visfatin protein in subcutaneous fat tissue was also confirmed by western blot analysis (Figure 1B). Visfatin expression was significantly increased in heart, liver and kidney compared to muscle, brain, and visceral fat. These expression results in WKY rats are in agreement with those reported by Revollo *et al.* (2007) in the mouse.

To test possible autocrine effects of visfatin (*iNampt*) on glucose uptake and insulin sensitivity in liver cells, we analyzed whether down regulation of visfatin expression by RNAi could affect glucose incorporation into Fao hepatocytes and how this will be modified in the presence of insulin. The efficiency of the silencing of the visfatin gene expression by RNAi was evaluated at the protein level by western blot analysis and at the mRNA level by qRT-PCR. Experiments included untreated control cells, and as a negative control, cells transfected with a nonsense sequence. Figure 1C and 1D shows significantly decreased levels of both RNA and protein in transfected Fao hepatocytes confirming significant down regulation of visfatin. The role of visfatin in cellular glucose uptake was analyzed by measuring incorporation of radioactively labeled ¹⁴C-glucose into Fao hepatocytes in the presence or absence of insulin in the incubation media. The results shown in Figure 2A demonstrate that Fao hepatocytes with down regulated visfatin expression exhibited significantly reduced levels of cellular glucose uptake and were insulin resistant when the incremental increase in glucose incorporation after insulin stimulation was significantly lower compared to control hepatocytes (0.6 ± 0.2 versus 2.2 ± 0.5 nmol/g/2h, $P=0.02$). These results indicate that autocrine effects of visfatin may play an important role in regulating insulin sensitivity in the liver. It can be hypothesized that insulin sensitizing effects of visfatin are related to its essential role in the NAD biosynthetic pathway. Accordingly, we also tested whether RNAi induced down regulation of visfatin affects NAD biosynthesis. As can be seen in Figure 2B, NAD biosynthesis rate was significantly reduced in the Fao hepatocytes with down regulated

levels of visfatin. These results provide evidence that visfatin exhibits important autocrine effects on sensitivity of liver cells to insulin action possibly through its effects on NAD biosynthesis.

Acknowledgments

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Figure legend

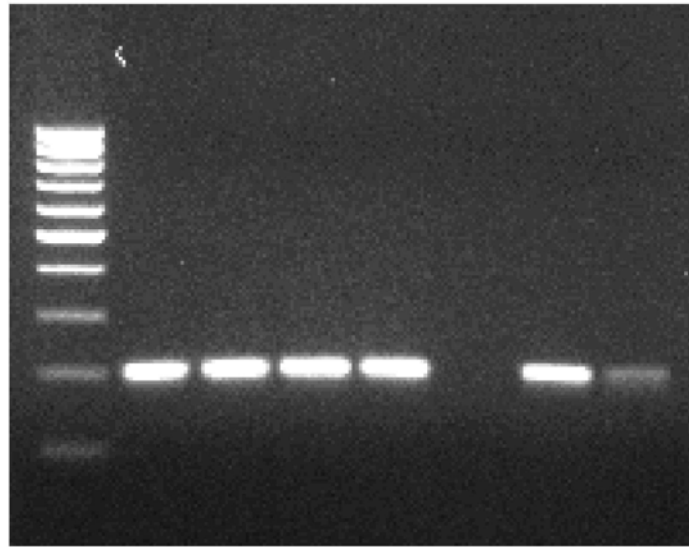
Figure 1 Expression of visfatin in tissues isolated from WKY rats. **A.** Agarose gel electrophoresis of visfatin-specific PCR products after reverse transcription from rat tissue mRNA. M - 1Kb DNA Ladder, 1 - heart, 2 - brain, 3 - kidney, 4 - liver, 5 – subcutaneous fat, 6 - striated muscle, 7 - visceral fat. **B.** Western blot analysis of tissue protein extracts. Protein visfatin was immunodetected by a polyclonal antibody using chemiluminescent detection. 1 - heart, 2 - liver, 3 - kidney, 4 - subcutaneous fat, 5 - striated muscle, 6 - visceral fat, 7 - brain. **C.** Relative quantification of visfatin gene expression in Fao hepatocytes following qRT-PCR analysis being determined in triplicates. Each sample was normalized to the expression of the house keeping gene. T - cells transfected with pSilencer containing sequence interfering with visfatin mRNA expression; NC - negative control, cells transfected with pSilencer-4.1 containing nonsense sequence; C - control cells. Results are expressed as means \pm SEM. * denotes $P < 0.05$ by Student t test. **D.** Western blot analysis of Fao hepatocyte cell lysates. Expression of visfatin and GAPDH (as a loading control) was immunodetected by polyclonal antibodies. Cell lysates were prepared from cells harvested after G418 selection. qRT-PCR, western blot analyses and RNAi were performed as described previously (Kontrová *et al.* 2007). Briefly, a Fao rat hepatocyte cell line was grown in HAM:F12 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂, 95% air incubator. Plasmids were prepared by cloning sequences complementary to rat PBEF-1 (GenBank BC085681): sense 5'-GATCCGGTTACTCACTATAAACAATTCAAGAGATTGTTATAGTGAGTAACCTTA-3' and antisense 5'-AGCTTAAGGTTACTCACTATAAACAATCTCTTGAATTGTTTATAGTGAGTAACCG-3' into *Bam*HI and *Hind*III sites in pSilencerTM 4.1-CMV-neo vectors (Ambion). After transfection, the cells were incubated with

DNA:FuGeneHD mixture for 48 h and 2 days in medium with 50 µg/ml of antibiotic G418.

Transfected cells were selected in F12:HAM containing 10% FBS and 120 µg/ml of G418.

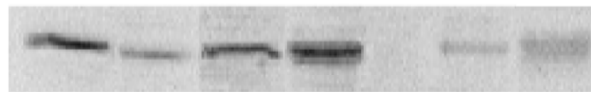
Figure 2 A. Basal and insulin stimulated glucose uptake by Fao hepatocytes being determined in triplicates. Hepatocytes with normal expression of visfatin exhibited significantly increased both basal and insulin stimulated glucose incorporation as well as incremental glucose incorporation after insulin stimulation. Glucose uptake by Fao hepatocytes was performed as described previously (Kontrová *et al.* 2007). **B.** Rates of NAD biosynthesis in Fao hepatocytes determined using the fluorescence method. C - control cells; T - cells transfected with pSilencer-4.1 containing sequence interfering with visfatin mRNA expression. Results being determined in triplicates are expressed as means ± SEM. * and ** denote P<0.05 and P<0.0005 by Student t test, respectively. Rate of NAD synthesis was measured by a fluorescence method. The reaction was performed in a 96-well microplate at 37°C with gentle shaking. Each well contained 200 µl of cell lysate in a reaction buffer (50 mM Tris-HCl, pH 8.8, 20 mM MgCl₂ and 50nM protease inhibitors), alcohol dehydrogenase from yeast (20 U) and substrate mixture (5 mM 5-phospho-α-D-ribose-1-diphosphate, 4 mM ATP, 50 mM nicotinamide, 1% ethanol). NADH fluorescence was measured for 16 h with a Fluoroskan Ascent FL type 374 fluorimeter (Thermo Electron Corporation) using a filter with an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

A.

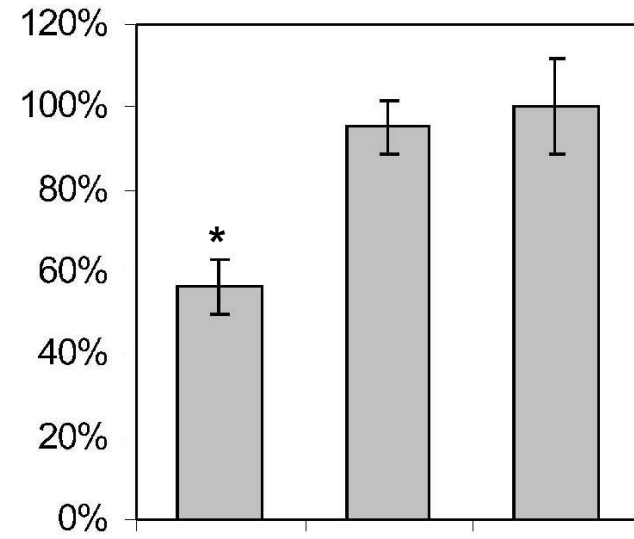


M 1 2 3 4 5 6 7

B.

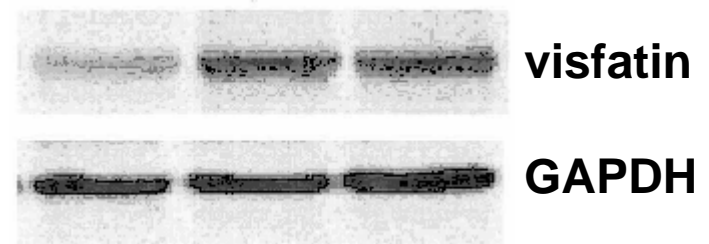


C.



T (RNAi) NC C

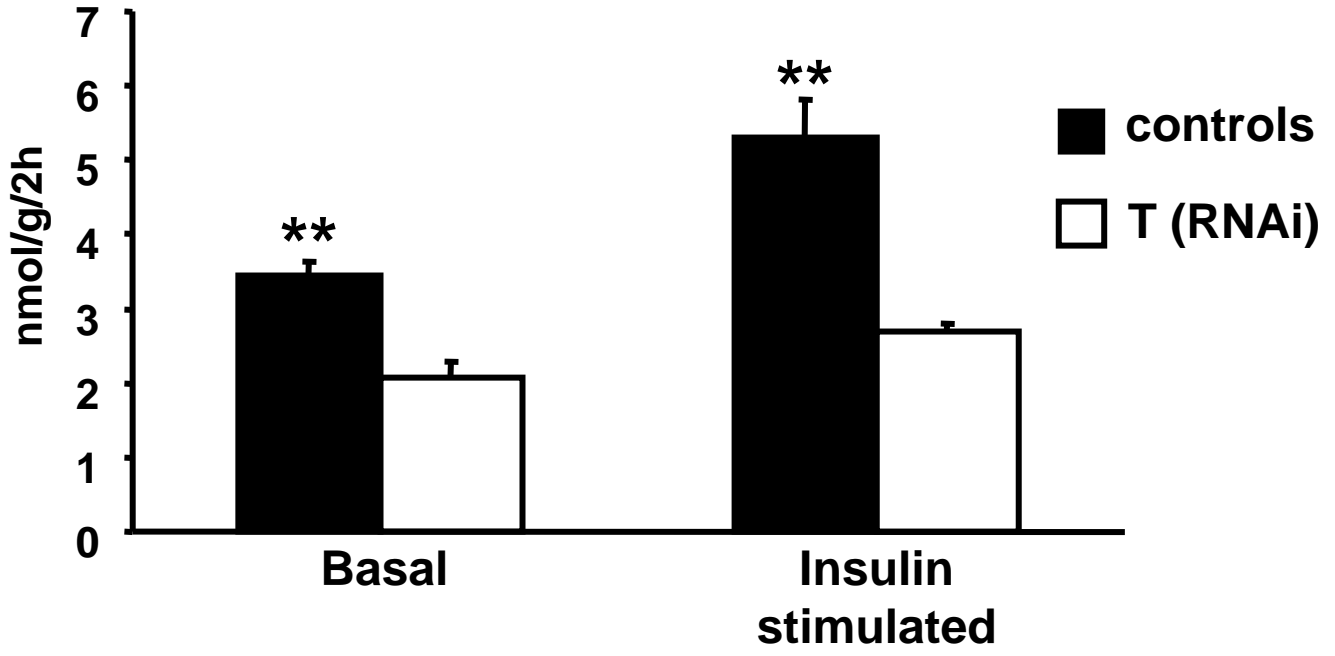
D.



visfatin

GAPDH

A.



B.

