Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise

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Frydelund-Larsen L, Akerstrom T, Nielsen S, Keller P, Keller C, Pedersen BK. Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise. Am J Physiol Endocrinol Metab 292: E24–E31, 2007. First published July 25, 2006; doi:10.1152/ajpendo.00113.2006.—Visfatin [pre-β-cell colony-enhancing factor (PBEF)] is a novel adipokine that is produced by adipose tissue, skeletal muscle, and liver and has insulin-mimetic actions. Regular exercise enhances insulin sensitivity. In the present study, we therefore examined visfatin mRNA expression in abdominal subcutaneous adipose tissue and skeletal muscle biopsies obtained from healthy young men at time points 0, 3, 4.5, 6, 9, and 24 h in relation to either 3 h of ergometer cycle exercise at 60% of V̇O_{\text{max}} or rest. Adipose tissue visfatin mRNA expression increased threefold at the time points 3, 4.5, and 6 h in response to exercise (n = 8) compared with preexercise samples and compared with the resting control group (n = 7, P = 0.001). Visfatin mRNA expression in skeletal muscle was not influenced by exercise. The exercise-induced increase in adipose tissue visfatin was, however, not accompanied by elevated levels of plasma visfatin. Recombinant human IL-6 infusion to mimic the exercise-induced IL-6 response (n = 6) had no effect on visfatin mRNA expression in adipose tissue compared with the effect of placebo infusion (n = 6). The finding that exercise enhances subcutaneous adipose tissue visfatin mRNA expression suggests that visfatin has a local metabolic role in the recovery period following exercise.

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The presence of low-grade, chronic, systemic inflammation is linked to an increased risk of developing cardiovascular disease and type 2 diabetes (8, 21). Regular exercise induces anti-inflammatory effects (9, 23), improves insulin sensitivity (3, 4), and thus offers protection against these conditions. Extensive evidence suggests that adipokines secreted from adipocytes, such as adiponectin, tumor necrosis factor-α (TNF-α), leptin, resistin, and adipin, play important roles in normal metabolic homeostasis and in the development of the metabolic syndrome (10).

Recently, visfatin, a new adipokine, was identified in visceral fat depots (13). Visfatin corresponds to a protein previously identified as pre-β-cell colony-enhancing factor (PBEF), which is expressed in adipose tissue, skeletal muscle, bone marrow, liver, and lymphocytes (13, 30). PBEF was earlier described as a growth factor enhancing the effect of IL-7 and stem cell factor on early-stage β-cells (30). Visfatin has insulin-like metabolic effects in various rodent models of insulin resistance and obesity in vivo (13). The metabolic effects of visfatin are apparently mediated by the binding to and activation of the insulin receptor (13). Consistent with its insulin-mimetic effects, treatment of adipocytes and muscle cells with recombinant visfatin increases basal glucose uptake and inhibits release of glucose from hepatocytes in vitro (13). However, the predominant expression of visfatin in visceral fat described by Fukuhara et al. (13) has been questioned by the finding of equivalent expression in subcutaneous and visceral adipose tissue (2, 19).

Several metabolic genes are activated in contracting skeletal muscles (15, 26). Evidence for cross talk between muscle and adipose tissue is provided by the finding of gene activation in abdominal subcutaneous fat during leg muscle work (14). Visfatin is expressed by both adipose tissue and skeletal muscle; however, its regulation has not been investigated. Given that regular exercise enhances insulin sensitivity, and given that visfatin enhances glucose uptake in myocytes and adipocytes, we hypothesized that visfatin is regulated in skeletal muscle and adipose tissue by exercise. In this study, we examined the effect of exercise on visfatin mRNA levels in human abdominal subcutaneous adipose tissue and in skeletal muscle. As IL-6 is markedly increased during exercise and has several metabolic effects, including induction of lipolysis and enhancement of insulin sensitivity, when administrated to IL-6-deficient mice (12, 22, 34), we additionally examined whether an exercise-induced regulation of visfatin could be mediated by IL-6.

MATERIALS AND METHODS

Study 1: Regulation of Visfatin in Adipose Tissue and Skeletal Muscle by Exercise

Subjects. Fifteen men with mean (±SD) age, height, weight, and BMI of 24.9 ± 4 yr, 180.9 ± 1 cm, 82.0 ± 8 kg, and 24.9 ± 2 kg/m², respectively, participated in this study. All subjects had a negative medical history, and physical examination revealed no abnormalities. Eight subjects exercised and seven subjects rested. There was no difference between the two groups with regard to age, weight, height, BMI, or maximal oxygen uptake (V̇O_{\text{max}}).

Experimental procedures. Cycle ergometer exercise was chosen as the mode of exercise in this study, since this type of exercise is mainly concentric and induces minimal muscle damage and subsequent inflammation. The subjects performed two incremental maximal exercise tests to determine V̇O_{\text{max}} on a cycle ergometer (Monark 839E; Monark, Varberg, Sweden). The first one, a familiarization trial, was performed 5 days prior to the first experimental day; the second test
was performed 2 days later. On the experimental day, subjects arrived at 0700 after an overnight fast. The subjects rested for ~10 min in the supine position, after which a venous catheter was placed in an antecubital vein. Subsequently, the subjects performed 3 h of cycling at 60% \( \dot{V}O_2 \text{max} \) followed by 6 h of recovery. Muscle and adipose tissue biopsies were obtained from the vastus lateralis and from the subcutaneous abdomen, respectively, prior to the exercise (0 h), immediately after exercise (3 h), and at 4.5, 6, 9, and 24 h, using the percutaneous needle biopsy technique with suction. Venous blood sampling for cytokines, insulin, and metabolites was drawn at baseline (0 h) and at 1, 2, 3, 4.5, 6, 9, and 24 h. The following day, they reported to the laboratory after an overnight fast, and a blood sample and muscle and adipose tissue biopsies were taken (24-h biopsies). Control subjects rested in the laboratory for 9 h, reported to the laboratory the day after in a fasted state, and had biopsy samples taken at the same time points as during the exercise trial. Biopsy samples were immediately placed on an ice-cold glass plate, cleaned of connective tissue and blood, and frozen in liquid nitrogen for further analysis.

**Study 2: Regulation of Visfatin mRNA Expression in Adipose Tissue by recombinant human IL-6**

**Subjects.** This study was part of a previous published investigation (16, 17). In short, 12 men, with mean (±SD) age, height, weight, and BMI of 26.0 ± 1 yr, 80.0 ± 4 kg, 185.0 ± 2 cm, and 23.5 ± 1 kg/m², respectively, participated in the study. All subjects had a negative medical history, and physical examination revealed no abnormalities. Six subjects were infused with recombinant human (rh)IL-6, and six subjects were infused with placebo.

**Experimental procedures.** On the day of the experiment, subjects reported to the laboratory at 0800 following an overnight fast. A peripheral catheter was placed in an antecubital vein for blood sampling, and one was placed in a contralateral vein for infusion with either rhIL-6 or vehicle 20% human albumin. rhIL-6 was infused for 3 h at a rate of 5 μg/h (Sandoz, Basel, Switzerland) in a volume of 25 ml/h administered in 20% human albumin (Statens Serum Institut, Copenhagen, Denmark). Six subjects were infused with vehicle 20% human albumin for 3 h. Adipose tissue biopsies were obtained prior to the infusion (0 h), at 1 and 2 h, immediately after the infusion (3 h), and at 5 and 8 h. Venous blood sampling for cytokines, insulin, and metabolites were collected at baseline (0 h) and at 3, 5, and 8 h.

**Ethics**

The subjects were given both oral and written information about the experimental procedures before they gave their written informed consent. All studies were approved by the Copenhagen and Frederiksberg Ethics Committee, Denmark, and were performed according to the Declaration of Helsinki.

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**Fig. 1.** A: visfatin mRNA expression in adipose tissue in exercising subjects (solid lines) and in resting control subjects (gray, dotted lines) in response to exercise (\( n = 8 \) and 7). Data are expressed as fold change from preexercise (Pre) values. B: average adipose tissue visfatin mRNA expression in the exercise group (filled bars) and in the resting control group (gray bars) (\( n = 8 \) and 7, respectively). Data are expressed as fold change from Pre values (geometric means ± SE). There was an increase in visfatin mRNA levels (\( P = 0.001 \)) in exercising persons compared with resting persons. Visfatin mRNA levels were increased at the following time points: immediately after the exercise bout (Post) and at 4.5 and 6 h. *Differences between groups; §difference from Pre value.
Analysis of Blood Samples

Blood samples were drawn into tubes containing EDTA and spun immediately at 2,500 × g for 15 min at 4°C. EDTA plasma was stored at −80°C until analysis. Plasma visfatin concentrations were measured with a human visfatin (COOH-terminal) enzyme immunometric assay (Phoenix Pharmaceuticals, Belmont, CA, catalog no. EK-003-80). The sensitivity for this assay is 0.5–1 ng/ml, and the intra- and interassay coefficients of variation (CVs) are 5 and 12%, respectively. Plasma IL-6 levels were measured with high-sensitivity ELISA kits (catalog no. HS600B; R&D Systems, Minneapolis, MN). The sensitivity is 0.094 pg/ml, and the intra- and interassay CVs are 6.9 and 9.6%, respectively. This kit does not distinguish between soluble and receptor-bound IL-6 and therefore gives a measurement of the total IL-6 content in the sample. Plasma insulin was measured using an ELISA kit (catalog no. K6219; DakoCytomation, Cambridgeshire, UK), with a sensitivity of 3 pmol/l and intra- and interassay CVs of 7.5 and 9.3%, respectively. The plasma levels of epinephrine were measured by the 2 CAT EIA kit from Lanor Diagnostika Nord (catalog no. BA 10-1500; Nordhorn, Germany). The sensitivity is 0.06 nmol/l and the intra- and interassay CVs are 15.0% and 13.2%, respectively. All the samples were run in duplicates with the samples from each subject on the same ELISA plate. Blood glucose was determined by COBAS (Fara, Roche) analysis on plasma.
**RNA Isolation and Reverse Transcription**

Total RNA was isolated from skeletal muscle and adipose tissue with TRIZol (Life Technology), as described by the manufacturer. Two micrograms of RNA were reverse transcribed in a 100-μl reaction according to the manufacturer’s instructions, using random hexamer primers (Applied Biosystems, Taqman reverse transcription reagents). The reactions were run in a PerkinElmer GeneAmp PCR system 9700 (PE Biosystems) with conditions 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

**Real-Time PCR Analysis**

Real-time PCR was performed on an ABI PRISM 7900 sequence detector (PE Biosystems). Each assay included (in triplicate) a cDNA standard curve of five serial dilution points (ranging from 1 to 0.01), a no-template control, a no-reverse transcriptase control, and 50 ng (12 ng for 18S rRNA and GAPDH) of each sample cDNA for PBEF and 150 ng for IL-6. The amplification mixture was made from 17.5 μl of 2X TaqMan Universal MasterMix, 1.75 μl of 20X TaqMan probe, and primers assay reagents, 7.5 μl of the cDNA preparation, and 8.25 μl of water to give a final volume of 35 μl. The primers and probes for PBEF (catalog no. Hs00237184_m1), 18S rRNA (catalog no. Hs99999901_s1), and GAPDH (catalog no. Hs99999905_m1) were predeveloped TaqMan probes, and primer sets from Applied Biosystems (AB). The IL-6 primers and probe sequences used were obtained from Starkie et al. (31). All assay reagents were from AB.

The amplification mixtures were amplified according to standard conditions using 50 cycles. The relative concentrations of PBEF, IL-6, and the endogenous controls 18S rRNA and GAPDH were determined by plotting the threshold cycle (CT) vs. the log of the serial dilution points. The relative expressions of PBEF and IL-6 were subsequently determined after normalization to the endogenous control. The levels of GAPDH mRNA in adipose tissue and skeletal muscle were not influenced by the exercise protocol, and the levels of 18S rRNA were not affected by rhIL-6 infusion (data not shown). For PBEF in adipose tissue, a slope of ~3.85 and an R² value of 1.0 were obtained. For PBEF in skeletal muscle, the slope was ~3.61 and the R² value 0.99. For IL-6 in adipose tissue, the slope was ~3.2 and the R² value 0.99.

The corresponding 18S rRNA values in adipose tissue and skeletal muscle, respectively, were ~3.10 and ~3.00 for slope and 1.0 for the R² values. For GAPDH in adipose tissue, the slope was ~3.23 and the R² value was 1.0. The y-intercept on the standard curves generated represented the Cₚ value for 50 ng of sample PBEF, which amounted to 26.5 and 24.6 in adipose tissue and skeletal muscle, respectively. The Cₚ value for 150 ng of sample IL-6 amounted to 35.1 in adipose tissue. The Cₚ values for 12 ng of 18S rRNA were 14.5 in adipose tissue and 12.0 in skeletal muscle. For 12 ng of GAPDH the Cₚ value was 18.7.

**Statistics**

All data were log transformed to reach a normal distribution and are presented as geometric means ± SE or as 95% confidence intervals (CI). The area under the curve over the 24-h period was calculated to evaluate the effect of exercise on visfatin mRNA expression over time and between groups. The area under the curve was calculated on the basis of the trapezium rule. For IL-6, a one-way ANOVA was used to analyze changes over time. For analysis of plasma data, a two-way repeated-measures ANOVA was used to detect changes over time or between groups. Post hoc analyses (Bonferroni-adjusted t-test) were performed to identify specific differences across time or between groups. Differences were considered significant at P < 0.05. Statistical calculations were performed using SYSTAT 8.0 (Richmond, CA).

**RESULTS**

**Effect of Exercise on Visfatin mRNA in Adipose Tissue, Skeletal Muscle, and Plasma**

Abdominal adipose tissue visfatin mRNA increased three-fold (95% CI 2.5- to 4.3-fold) in response to exercise compared with preexercise samples and compared with resting subjects (Fig. 1). A significant increase in visfatin mRNA was seen at three time points in the exercise group, 3 h (Post), 4.5 h, and 6 h, which were also significantly different from the resting control group (P = 0.001). In contrast, visfatin mRNA expression in skeletal muscle was unaltered by exercise (Fig. 2).

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<th>Table 1. Plasma levels of visfatin, IL-6, insulin, and epinephrine in response to exercise or rest</th>
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<td><strong>Exercise</strong></td>
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<td>Visfatin, ng/ml</td>
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Values are geometric means (95% confidence interval in parentheses); n = 8 and 7, respectively. NA, not applicable. *Significant difference between groups; §significant difference from Pre values.
It appears from Table 1 that plasma levels of visfatin did not change in response to exercise. Plasma IL-6 levels increased during exercise to reach a 33-fold increase (95% CI 22- to 50-fold) at the end of the exercise bout. Epinephrine levels increased 10-fold (95% CI 6- to 17-fold), whereas plasma-insulin levels and glucose levels decreased slightly.

**Effect of Exercise on IL-6 mRNA in Adipose Tissue**

In accordance with present findings, adipose tissue IL-6 mRNA increased 10-fold (95% CI 2.5- to 17.5-fold) in response to exercise compared with preexercise samples ($P = 0.01$; Fig. 3) (14). A significant increase in IL-6 mRNA was seen at four time points: 3 h (Post), 4.5 h, 6 h, and 9 h.

**Effect of rhIL-6 on Visfatin in Adipose Tissue**

Given that exercise enhances IL-6 mRNA in adipose tissue, we examined whether the effect of exercise on adipose tissue visfatin could be mediated by IL-6. Adipose tissue biopsies were obtained in relation to rhIL-6 infusion or vehicle. Plasma IL-6 concentrations during rhIL-6 infusion were 4.5 (2.3–9.2), 98.5 (68.8–143.8), 6.4 (5.0–8.3), and 6.9 pg/ml (4.6–10.4) at time points: 0 h (Pre), 3 h (Post), 5 h, and 8 h, thus reaching levels comparable to/above that obtained in response to exercise (17). However, rhIL-6 infusion had no effect on the level of visfatin mRNA (Fig. 4).

**DISCUSSION**

Initially, visfatin was described as a protein preferentially produced in visceral adipose tissue (13). However, recent studies in rodents (19) and humans (2) find no difference with regard to expression of visfatin by visceral and subcutaneous fat. The main finding of the present study was that visfatin is threefold upregulated at the mRNA in abdominal subcutaneous adipose tissue by acute exercise. However, visfatin in skeletal muscle was not regulated by exercise.

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**Fig. 3.** A: IL-6 mRNA expression in adipose tissue in exercising subjects. Data are expressed as fold change from Pre values. B: average adipose tissue IL-6 mRNA expression in the exercise group. Data are expressed as fold change from Pre values (geometric means ± SE; $n = 8$ each). IL-6 mRNA levels increased significantly compared with Pre values ($P = 0.03$).
Visfatin may contribute to enhanced insulin sensitivity. Acute administration of recombinant visfatin to mice leads to a reduction of plasma glucose in a dose-dependent fashion, and chronic elevation of plasma visfatin attenuated both plasma glucose and insulin levels (13). Moreover, treatment with the peroxisome proliferator-activated receptor-γ agonist rosiglitazone resulted in increased visceral adipose tissue mRNA in a rat model, suggesting that the enhanced insulin sensitivity is mediated in part by visfatin (6). Type 2 diabetic patients have elevated levels of plasma visfatin compared with nondiabetic individuals, suggesting impaired visfatin signaling or dysregulation of visfatin expression in these patients (5). However, in contrast to the original findings, visfatin mRNA levels in visceral fat and subcutaneous fat were not correlated with markers of insulin resistance after adjustment for BMI in a recent cohort study (2). The biological role of visfatin in humans is therefore less clear. On the basis of the present finding that the visfatin mRNA level in subcutaneous adipose tissue is regulated by exercise, we speculate whether visfatin in subcutaneous tissue may have a metabolic role in the recovery period following exercise.

It is unclear whether visfatin is secreted into the systemic circulation, as the primary visfatin amino acid sequence does not contain a signal peptide (30). Previous reports have found visfatin to be located primarily in the cell nucleus and in the cytoplasm (18), questioning an endocrine role of visfatin. However, visfatin may be secreted via an alternative pathway (1, 29). In support of this idea, Samal et al. (30) detected
visfatin in the conditioned medium of activated lymphocytes, and, in agreement with our findings, circulating levels of visfatin are found in healthy humans (2, 5, 13). In a recent large-scale study, plasma levels of visfatin were correlated with visfatin mRNA levels in visceral but not subcutaneous adipose tissue (2). Exercise-induced changes in adipose tissue visfatin mRNA levels were not reflected in changes in the plasma levels in the present study. It is therefore most likely that the role of visfatin in relation to acute exercise is to mediate paracrine effects rather than mediating an endocrine effect.

During exercise, IL-6 is expressed by working skeletal muscle (25, 32) and adipose tissue (14, 16), and these organs contribute to the increased circulating levels of IL-6. Thus, exercise-induced IL-6 is believed to work in an endocrine manner (11, 12). An in vitro study demonstrated that high concentrations of IL-6 is a negative regulator of visfatin gene expression in murine adipocytes (20). The human in vivo relevance of this finding is not clear, since obese individuals are characterized by elevated circulating levels of both visfatin (13) and IL-6 (28, 35). In the present study, rhIL-6 infusion to mimic the exercise-induced rise in IL-6 did not affect the level of adipose tissue visfatin mRNA expression. Thus, we did not find support for a role of IL-6 in the regulation of adipose tissue visfatin levels. However, when the adipose tissue visfatin mRNA response for each subject was compared with the increase in their adipose tissue IL-6 mRNA, it appeared that those subjects with the highest increase in adipose tissue visfatin mRNA were those with the highest IL-6 mRNA response. IL-6 has been demonstrated to have a lipolytic effect, thus possibly playing a role in the mobilization of energy such as free fatty acids in response to exercise (22, 34). During exercise, the combination of falling insulin concentrations and increasing catecholamines, growth hormone, and cortisol are the primary regulators of adipose tissue lipolysis. We therefore speculate whether the exercise-induced increase in adipose tissue visfatin mRNA may be mediated by epinephrine or by epinephrine-induced changes in free fatty acids.

Sex differences in the relative oxidation of carbohydrates and lipids in response to acute exercise have been demonstrated (24, 27, 33). Only male subjects participated in the present study, and we therefore cannot exclude the possibility that visfatin might be differently regulated in response to exercise in males and females. In two recent studies investigating the mRNA levels of visfatin in subjects with a wide range of obesity, body fat distribution, insulin sensitivity, and glucose tolerance, no differences between males and females were found (2, 5). However, when Berndt et al. (2) studied the relationship between plasma visfatin, BMI, and body fat, they found a correlation in males only, suggesting that there might be sex differences (2).

In a recent study investigating the cellular source of visceral fat adipokines in obese individuals, visfatin was expressed mainly by accumulating macrophages (7). Adipose tissue biopsies may contain various cells types besides adipocytes e.g., white blood cells. As the subjects in this study were lean (mean BMI 24.9 kg/m²), macrophages are expected to constitute only a small fraction of the adipose tissue (36). However, on the basis of the present study we cannot come to a conclusion on the cellular origin of visfatin in the adipose tissue biopsies.

In summary, exercise enhances visfatin mRNA levels in abdominal subcutaneous adipose tissue. This finding suggests that visfatin in subcutaneous adipose tissue may have a metabolic role in the recovery period following exercise. The increased mRNA expression of adipose tissue visfatin was not accompanied by elevated levels of plasma visfatin, indicating that subcutaneous adipose tissue visfatin may act in a paracrine rather than an endocrine manner.

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