Identification of Nesfatin-1 in Human and Murine Adipose Tissue: A Novel Depot-Specific Adipokine with Increased Levels in Obesity

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Nesfatin-1 is a recently identified anorexigenic peptide derived from its precursor protein, non-esterified fatty acid/nucleobindin 2 (NUCB2). Although the hypothalamus is pivotal for the maintenance of energy homeostasis, adipose tissue plays an important role in the integration of metabolic activity and energy balance by communicating with peripheral organs and the brain via adipokines. Currently, no data exist on nesfatin-1 expression, regulation, and secretion in adipose tissue. We therefore investigated NUCB2/nesfatin-1 gene and protein expression in human and murine adipose tissue depots. Additionally, the effects of insulin, dexamethasone, and inflammatory cytokines and the impact of food deprivation and obesity on nesfatin-1 expression were studied by quantitative RT-PCR and Western blotting. We present data showing NUCB2 mRNA (P < 0.001), nesfatin-1 intracellular protein (P < 0.001), and secretion (P < 0.01) were significantly higher in sc adipose tissue compared with other depots. Also, nesfatin-1 protein expression was significantly increased in high-fat-fed mice (P < 0.01) and reduced under food deprivation (P < 0.01) compared with controls. Stimulation of sc adipose tissue explants with inflammatory cytokines (TNF-α and IL-6), insulin, and dexamethasone resulted in a marked increase in intracellular nesfatin-1 levels. Furthermore, we present evidence that the secretion of nesfatin-1 into the culture media was dramatically increased during the differentiation of 3T3-L1 preadipocytes into adipocytes (P < 0.001) and after treatments with TNF-α, IL-6, insulin, and dexamethasone (P < 0.01). In addition, circulating nesfatin-1 levels were higher in high-fat-fed mice (P < 0.05) and showed positive correlation with body mass index in human. We report that nesfatin-1 is a novel depot-specific adipokine preferentially produced by sc tissue, with obesity- and food deprivation-regulated expression. (Endocrinology 151: 3169–3180, 2010)

Obesity is a leading cause of morbidity and mortality worldwide (1) and is known to arise from an imbalance between energy intake and expenditure. The hypothalamus is the key center for integration of long-term energy balance and is a rich source of satiety regulatory peptides. Many of these peptides are also found in peripheral sites such as the gut and adipose tissue in which they play key physiological roles in body weight homeostasis and contribute to the pathophysiology of insulin resistance and its associated metabolic complications in obesity and diabetes (2–4).

Nesfatin-1 is a recently described anorexigenic peptide derived from a precursor molecule NUCB2, which contains an amino acid sequence that is highly conserved be-
tween human and rodents (5). Posttranslational processing of nucleobindin 2 (NUCB2) by prohormone convertase produces three cleavage products, namely nesfatin-1 (1–82), nesfatin-2 (85–163), and nesfatin-3 (166–396). A number of strands of evidence implicated nesfatin-1 as an anorexigenic molecule. First, NUCB2/nesfatin-1 is abundantly expressed in several regions of the hypothalamus that play key roles in controlling food intake (6). Second, NUCB2 mRNA levels are reduced during fasting and significantly increase on refeeding in the rat paraventricular nucleus (7). Finally, nesfatin-1 has been shown to reduce food intake in rodents when administered either centrally or peripherally (5, 8). Also, nesfatin-1 has been demonstrated to cross the blood-brain barrier bidirectionally in a nonsaturable way (9), raising the possibility that peripheral nesfatin-1 might penetrate the brain and influence appetite (8).

Evidence for a peripheral source of nesfatin-1 has been provided by the observation that NUCB2 mRNA is expressed in gastric endocrine cells (10). It is likely, that another potential source of peripheral nesfatin-1 is adipose tissue, which is well established as a major endocrine organ and a rich source of molecules involved in the central regulation of satiety (11–13).

With the aforementioned, we investigated whether adipose tissue was a source of NUCB2/nesfatin-1. Moreover, we aimed to determine whether food deprivation and obesity influenced NUCB2/nesfatin-1 mRNA and protein expression. Additionally, we studied the profile of NUCB2 expression at different stages of adipocyte differentiation. Furthermore, regulation of nesfatin-1 in adipose tissue depots and 3T3-L1 adipocytes by factors known to be altered in obesity was investigated. Finally, circulating nesfatin-1 was assessed in human subjects across a range of body mass index (BMI).

Materials and Methods

Biochemical reagents

Nesfatin-1 peptide was obtained from Phoenix Pharmaceuticals (Belmont, CA). TNF-α, IL-6, dexamethasone, insulin, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, UK). Agarose was obtained from MBI Fermentas Co. Ltd. (Gillingham, Dorset, UK). Western blotting detection reagents were purchased from Bio-Rad Laboratories Ltd. (Hertfordshire, UK). Polyvinylidene difluoride (PVDF) membrane was purchased from Amersham Biosciences; all primers were acquired from TAGN (Newcastle, UK).

Antibodies

Antinesfatin-1 (1–82) and antinesfatin-3 antibodies was supplied by Phoenix Pharmaceuticals, and mouse anti-β-actin antibody was purchased from Abcam (Cambridge, UK). Polyclonal horseradish peroxidase-conjugated goat antirabbit, and anti-mouse immunoglobulin/horseradish peroxidase was purchased from DakoCytomation (Glostrup, Denmark). A polyclonal fluorescence goat antirabbit fluorescein isothiocyanate was obtained from Invitrogen, (Paisley, UK).

Cell culture and differentiation

3T3-L1 cells were purchased from American Type Culture Collection (Manassas, VA). 3T3-L1 preadipocytes were maintained and differentiated as previously described (14, 15). 3T3-L1 cells were cultured in DMEM (Invitrogen) supplemented with 10% bovine growth serum at 37°C. For adipocyte differentiation, confluent cells were cultured for 2 d in differentiation media containing 1 μm dexamethasone, 0.5 mm methylisobutyrylacetone, and 0.860 μm insulin (Cayman Chemical, Ann Arbor, MI). From d 2, media containing only insulin (0.860 μM) and dexamethasone (1 μM) were used for the next 10 d. At d 0, 2, 4, 8, and 12 of differentiation, the cells were harvested for RNA isolation and Western blot analysis. Corresponding conditioned media were collected and stored at −80°C.

Animals, diets, and feeding procedures

Adult male C37BL/6 wild-type mice (n = 30) were obtained from Harlan UK, Ltd. All mice were housed individually and maintained under pathogen-free conditions with controlled temperature and humidity and a 12-h light (0700–1900 h) cycle as well as free access to standard mice diet and water. Both standard diet and high-fat diet were obtained from RMI, Dietex International Ltd., Essex, UK: standard diet consisted of 70% carbohydrate, 10% fat, and 20% protein, with an energy density of 3.85 kcal/g, whereas the high-fat diet consisted of 40% fat (31% lard and 9% corn oil), 45% carbohydrate (15.2% corn starch, 5% cellulose, and 20% sucrose), and 15% protein (casein), with an energy density of 4.71 kcal/g.

Food deprivation studies

One group of mice was deprived of food for 24 h before they were killed, whereas the control group was fed on standard diet. All animals were culled by cervical dislocation at 0900 h and sc and omental (OM) adipose tissue were dissected and placed into sterile DMEM (Invitrogen) for explant culture studies and/or immediately snap frozen in liquid nitrogen and stored at −80°C. All procedures were performed in accordance with the U.K. Guidance on the Operation of Animals (Scientific Procedures) Act (1986).

Mouse primary adipose tissue explant culture

Adipose tissue organ explants were cultured using a modified method (16). Briefly, 1–3 g of adipose tissue was minced into 5–10 mg (~1 mm3) fragments, washed with a 230-μm mesh (Filter no. 60; Sigma-Aldrich), and rinsed with sterile PBS warmed to 37°C. Samples were then transferred to six-well plates (~50 mg/ml) containing 3 ml Media 199 (Invitrogen) supplemented with 100 μM gentamicin and 1% fetal calf serum (containing insulin at a concentration of 10−14 M) and cultured for 24 h with or without the addition of TNF-α, IL-6, insulin, or dexamethasone in a 37°C incubator under an atmosphere of 5% CO2-95% air.

Isolation of adipocytes from mouse adipose tissue

Adipocytes were isolated from adipose tissue as previously described, with slight modifications (17). Adipose tissue biopsies were chopped finely and adipocytes isolated by collagenase di-
gestion [Hanks’ balanced salt solution, containing 3 mg/ml collagenase (type II) and 1.5% BSA] in a shaking water bath at 37 C for 60 min. Cell suspensions were subsequently filtered through a 230-μm screen cup filter mesh no. 60 (Sigma). Mature adipocytes were separated from stromal vascular cells through an inert oil, bis(3,5,5 trimethylhexyl) phthalate, specific gravity 0.98 (Fluka Chemicals, Gillingham, Dorset, UK) by a method previously described (18). To the filtered suspension, 1 ml bis(3, 5, 5 trimethylhexyl) phthalate was added, which was then centrifuged for 5 min at 1500 rpm. The adipocytes form a layer on top of the oil, which has a lower density than the collagenase-digestion medium and a higher density than the adipocytes. The stromal vascular cells were treated with an erythrocyte lysis buffer and processed along with the isolated adipocytes for RNA extraction as described for adipose tissue explants.

**Human subjects**

For gene and protein expression studies, adipose tissue biopsies (sc and OM) were obtained (0800–1000 h) from adult female patients undergoing elective surgery during the early follicular phase of the menstrual cycle (d 3–5) for infertility investigation. Subjects were initially seen at the infertility clinic and then scheduled for laparoscopy to assess Fallopian tube(s) patency (n = 6, BMI 22.3–27.67 kg/m², age 27–38 yr). All subjects studied were nonsmokers and otherwise healthy. Patients had been fasted overnight before surgery and were not taking any medications for at least 6 months before the study, including oral contraceptive agents or hormone-replacement therapy. The study was approved by the local ethics committee, and all patients involved gave their informed consent in accordance with the guidelines in the Declaration of Helsinki.

For determination of circulating nesfatin-1, 20 subjects participated in the study (nine men; age 28–58 yr; BMI 21.39–38.10 kg/m²). Exclusion criteria were a history of diabetes, congestive heart failure, liver or kidney disease, malignancy, signs of inflammation, pregnancy, and any drugs influencing body weight like corticosteroids or contraceptives. The study was approved by the local ethics committee, and all subjects gave written informed consent. After an overnight fast, blood samples were collected and immediately centrifuged, and plasma samples frozen at −80 C until assayed. Nesfatin-1 was determined using a commercially available enzyme immunoassay (enzyme immunoassay kit; Phoenix Pharmaceuticals). Analyses were performed according to the manufacturer’s protocol for determination of nesfatin-1 in plasma.

**RT-PCR and quantification of mouse and human NUCB2 mRNA**

Total RNA was extracted using the QIAGEN RNasy minikit (West Sussex, UK), deoxyribonuclease treated, and reverse transcribed into cDNA using a reverse transcriptase kit according to the manufacturer’s instructions (Fermentas Life Sciences, York, UK). The sequences for the sense and antisense primers (respectively) were: human NUCB2 (175 bp) sense, 5′-GCGAGGA-CGTGTATCAGCTC-3′ and antisense, 5′-CTCTGCCCCACCTT-CATGTTGAC-3′; mouse NUCB2 (195 bp) sense, 5′-CTCTGG- GAAGCTGTTCCTATC-3′ and antisense, 5′-CGCTCTTTA-TTCTGCTCAG-3′; mouse β-actin (187 bp) sense, 5′-GTCGTA-TAGCCCAACACATGTC-3′ and antisense, 5′-TCTGCTGGAGG- GTGGAAGCTC-3′; human β-actin (216 bp) sense, 5′-AAA- GAGAGGACCACCTCCACTC-3′ and antisense, 5′-TACAT-GGCTGGGTTCTTGA-3′. SYBR Green real-time PCR was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany) using the primers listed above. PCR was performed using 2.5 μl cDNA in 5.5 μl PCR SYBR Green-1 Light Cycler master mix (Biogene, Cambridgeshire, UK) and 1 μl sense and antisense primers. A series of three dilutions for each cDNA was used to ensure linear amplification and to measure primer efficiency. Protocol conditions consisted of denaturation of 95 C for 60 sec, followed by 40 cycles of 94 C for 1 sec, 60 C for 8 sec, and 72 C for 15 sec, followed by melting-curve analysis. For analysis, expression of genes of interest were normalized against the expression of the housekeeping gene β-actin. Negative controls for all the reactions included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. The relative mRNA levels were expressed as a ratio using the 2-ΔΔct method for comparing relative expression results between treatments in real-time PCR (19). PCR products were stained with ethidium bromide and visualized by electrophoresis through 2% agarose gels. Sequencing of the PCR products confirmed the sequence identities (data not shown). NUCB2 gene expression was measured by real-time PCR, using 1 μg total RNA and oligo-deoxythymidine primers as reverse transcriptase primers.

**Western blotting and immunohistochemistry**

Protein lysates were prepared by adding equal amounts of radiodinmunoprecipitation assay buffer to disrupt cells/tissues, and resulting samples were quantified using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Samples were adjusted to equal amounts using Laemmli buffer, subsequently sonicated, and boiled. Samples were separated by SDS-PAGE (10% resolving gel) and transferred to PVDF membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The PVDF membranes were incubated with primary antibody for NUCB2/nesfatin-1 (Phoenix Pharmaceuticals) at a 1:2,000 dilution nesfatin-3 (Phoenix Pharmaceuticals) at 1:2,500 dilution and β-actin (Abcam) at 1:2,500 in Tris-buffered saline-0.1% Tween 20, and 5% BSA overnight at 4 C. The membranes were washed, incubated with a secondary antirabbit IgG (nesfatin-1 and nesfatin-3) and antimuscle IgG (β-actin) horseradish peroxidase-conjugated antibody (1:2000, Sigma-Aldrich) for 1 h at room temperature, and washed for 60 min with Tris-buffred saline-0.1% Tween 20. Antibody complexes were visualized using the ECL Plus chemiluminescence detection kit (GE Healthcare UK Ltd., Buckinghamshire, UK). The densities were measured using a scanning densitometer coupled to Scion Image scanning software (Scion Corp., Frederick, MD).

**Immunohistochemistry**

Mice were killed by cervical dislocation. Subcutaneous adipose tissue was postfixed for 2 h and stored in 30% sucrose/PBS solution overnight. Adipose tissue sections were cut at 3–5 μm and floated onto 3-aminopropyltriethoxy-silane-coated slides. Heat-induced antigen retrieval was used Tris-EDTA buffer (pH 7.8). After washing with PBS, paraformaldehyde (4%) fixation, and dehydration through series of alcohol, the slides were left to air dry at room temperature. Nonspecific binding was reduced by blocking with 10% BSA in PBS for 60 min at room temperature. Sections were incubated with polyclonal rabbit antibody against the C terminus of nesfatin-1 (1: 500 in PBS, Phoenix Pharmaceuticals) for 60 min at room temp-
perature. After three PBS washes for 5 min each, donkey anti-rabbit IgG-fluorescein isothiocyanate (Invitrogen) was added at 1:400 dilution for 1 h at room temperature. After three 5-min washes with PBS, the slides were mounted with Vectashield mounting medium containing 4′,6′-diamino-2-phenylindole counterstain (Vector Laboratories, Peterborough, UK). The slides were examined under an oil immersion objective (×40), and fluorescent signals were analyzed using a Zeiss Axioskope 2 microscope equipped with a cooled charge-coupled device camera (CoolSnap; Photometrics Ltd., Tucson, AZ) controlled by a Power Macintosh computer (Flextronics, Venray, Netherlands). After indirect immunofluorescent staining, no specific fluorescence was observed in cells treated with secondary antibody only. For each group, between 10 and 15 random fields were selected and examined.

ELISA

Human plasma nesfatin-1 levels were measured using a commercially available ELISA kit (Phoenix Pharmaceuticals, Belmont, CA), according to the manufacturer’s instructions. The assay has a detection limit of 0.78 ng/ml. Murine circulating nesfatin-1 and the secretion from adipose tissue explants were measured using a commercially available enzyme immunoassay (nesfatin-1 mouse and rat detection kit; Axxora, Nottingham, UK) according to the manufacturer’s instructions. The assay had a minimum detection limit of 0.3 ng/ml. All samples were analyzed in duplicates. Total protein content from sc and OM tissue explants were determined by BCA assay as per the manufacturer’s instructions. The BCA assay had a detection range of 100-5000 µg/ml. All the samples were analyzed in duplicates.

Statistical analyses

Data were analyzed by Mann–Whitney U test, ANOVA (post hoc analysis: Dunn’s test) according to the number of groups compared. P < 0.05 was considered significant. For Western immunoblotting experiments, the densities were measured using a scanning densitometer coupled to scanning software (Scion Image; Scion). Spearman correlations were used for calculation of associations between variables. P < 0.05 was considered significant. Spearman correlation was performed to detect associations between plasma nesfatin-1 and BMI.

Results

**NUCB2/nesfatin-1 in mouse and human adipose tissue and 3T3-L1 cells**

NUCB2 mRNA was detected in both mouse and human sc and OM adipose tissue and also in the 3T3-L1 cell line (Fig. 1, A and B, n = 4). Furthermore, Western blot analysis confirmed nesfatin-1 protein in these tissues (Fig. 1, C and D, n = 4). Immunohistochemical studies performed to identify subcellular localization of nesfatin-1 showed substantial positive staining in the periphery of adipocytes from sc adipose tissue and cytoplasm of 3T3-L1 cells (Fig. 1E). In addition to mRNA and protein expression, secretion of nesfatin-1 into conditioned media was determined by ELISA; significantly higher levels were noted from sc adipose tissue explants compared with OM adipose tissue in both murine and human explants (Fig. 1F; P < 0.01). With these observations in mind, further studies were conducted using sc adipose tissue explants and 3T3-L1 cells to investigate regulation of NUCB2/nesfatin-1.

**Site-specific expression of NUCB2 mRNA in murine and human adipose tissue**

Experimental analysis of site-specific expression of NUCB2 mRNA showed a significantly higher expression level in sc tissue compared with OM, perirenal, epicardial, and brown adipose tissue in mice (Fig. 2A; P < 0.001). This relative difference in site-specific NUCB2 expression in murine tissue was supported by the observation that human sc adipose tissue had significantly higher NUCB2 mRNA expression compared with OM adipose tissue (Fig. 2B; P < 0.001).

**Expression of NUCB2 in adipocytes and stroma-vascular cells**

To determine the cellular production of NUCB2/nesfatin-1 in adipocyte, adipocytes, and stroma-vascular cells were isolated from sc adipose tissue, with leptin as a control. Quantitative RT-PCR analysis showed a significantly higher level of NUCB2 mRNA expression in the adipocyte fraction compared with the stroma-vascular fraction (Fig. 2C; P < 0.05).

**NUCB2 mRNA and nesfatin-1 protein production and secretion during 3T3-L1 differentiation**

NUCB2 mRNA and nesfatin-1 protein were detected in both pre- and postdifferentiated 3T3-L1 cells. The expression of both NUCB2 mRNA and nesfatin-1 protein increased during the process of differentiation, with significantly increased mRNA expression being observed from d 2 onward (Fig. 3A; P < 0.001). Intracellular protein expression and also nesfatin-1 secretion into conditioned media were significantly increased throughout the process of differentiation (Fig. 3, B–D; P < 0.001), a profile mirroring that of the adipogenic marker peroxisomal proliferator-activated receptor (PPAR)\(^\gamma\). In addition to PPAR\(^\gamma\), other key adipocyte differentiation markers such as fatty acid binding protein-2 and preadipocyte factor-1 were also used as controls to assess 3T-L1 differentiation (Fig. 3, E and F). Interestingly, the mRNA and intracellular protein expression of NUCB2/nesfatin-1 (Fig. 3, A and B) continued to increase up to d 12 similar to that of the AP-2 (Fig. 3, E and F). The quantitative relative changes in NUCB2 mRNA is depicted in Fig. 3A, and intracellular nesfatin-1 protein expression and secretion are depicted in Fig. 3D.
Effect of food deprivation on NUCB2 mRNA and nesfatin-1 protein expression

Previous studies described altered hypothalamic NUCB2 mRNA expression during feeding and food deprivation (5). In view of this, we examined the effect of food deprivation on NUCB2 mRNA and nesfatin-1 protein expression in murine sc adipose tissue. In this study one group of mice was deprived of food for 24 h before they were killed, whereas the control group was fed a normal diet. The levels of NUCB2 mRNA and nesfatin-1
protein expression in sc adipose tissue in each group were examined by RT-PCR and Western blotting, respectively. Interestingly there was a significant decrease in NUCB2 mRNA (Fig. 4A; \( P < 0.01 \)) and nesfatin-1 protein expression levels (Fig. 4B; \( P < 0.01 \)) in sc adipose tissue of mice that were food deprived compared with those who were fed. These data are in agreement with reports from Oh-I et al. (5) showing that food deprivation induces a reduction in hypothalamic NUCB2 mRNA. These findings suggest that NUCB2 mRNA and nesfatin-1 protein expression in murine sc adipose tissue might be regulated by the tissue energy supply.

**Effect of a high-fat diet and obesity on nesfatin-1 protein expression and secretion**

Altered metabolic states, including obesity, can change the expression of adipose tissue-derived proteins. To investigate the impact of obesity on nesfatin-1 expression, mice were fed either a standard diet (control: 70% carbohydrate, 10% fat, and 20% protein) or a high-fat diet (40% fat, 45% carbohydrate, and 15% protein), as mentioned above. Mice were culled at 12 and 20 wk. Mice fed the high-fat diet had significantly higher levels of nesfatin-1 protein expression in adipose tissue at both 12 and 20 wk, compared with the control group (Fig. 4, C and D; \( P < 0.01 \)). In addition, the circulating levels of nesfatin-1 was significantly higher in mice fed a high-fat diet (4.7 ± 0.38 ng/ml) compared with mice fed a standard diet (3.1 ± 0.45 ng/ml; \( P < 0.05 \)).

**Effect of cytokines, insulin, and dexamethasone nesfatin-1 protein expression in 3T3-L1 cells**

Obesity and dysmetabolic states are associated with altered levels of circulating cytokines, insulin, and corticosteroids, which are known to regulate production of certain adipokines. We therefore sought to investigate whether these factors might regulate nesfatin-1 protein expression in 3T3-L1 cells. Cells were challenged with increasing concentrations of TNF-\( \alpha \), IL-6, insulin, and dexamethasone for a period of 24 h. Each of these treatments caused a dose-dependent increase in nesfatin-1 protein expression in the 3T3-L1 adipocytes (Fig. 5, A–D; \( P < 0.001 \)).
Ex vivo effects of cytokines, insulin, and dexamethasone on nesfatin-1 protein expression and secretion in sc adipose tissue explants

To confirm these results, we used an alternative experimental paradigm with an ex vivo model of sc adipose tissue explants. We examined both protein expression in tissue lysates and secretion into the conditioned culture media using the concentration of cytokine, insulin, or dexamethasone that showed the maximum response in the 3T3-L1 cells. We observed that nesfatin-1 protein production was significantly increased by insulin, dexamethasone (Fig. 5E; \( P < 0.001 \)), and also IL-6 and TNF-\( \alpha \) (Fig. 5F; \( P < 0.001 \)). Interestingly, this was accompanied by increased secretion of nesfatin-1 into the culture media in the groups treated with insulin, dexamethasone, and IL-6 (Fig. 5G; \( P < 0.01 \), \( P < 0.001 \)) but decreased in the TNF-\( \alpha \)-treated group (Fig. 5G; \( P < 0.05 \)).
Circulating nesfatin-1 levels in human plasma

Mean nesfatin-1 levels were 2.90 ± 0.30 ng/ml, with no significant difference between men (2.64 ± 0.35 ng/ml) and women (3.20 ± 0.47 ng/ml). There was a significant positive correlation between plasma nesfatin-1 and BMI ($r = 0.596$; $P = 0.004$; Fig. 6).

Discussion

NUCB2/nesfatin has been shown to be present in the hypothalamus in regions involved in appetite control (5). Oh-I et al. (5) used a subtraction-cloning assay of PPAR-γ activator-stimulated genes in SQ-5 cells to identify potential new appetite-regulating molecules and discovered NUCB2/nesfatin-1 to be a potent anorexigenic molecule. Given that many hypothalamic secreted molecules also exist in peripheral adipose tissue, the authors examined whether the genes cloned were also expressed in other tissue/cells. Interestingly, the authors found NUCB2 mRNA to be expressed in medulloblastoma (HTB185) and 3T3-L1 adipocyte cells. Furthermore, recent studies have shown expression of NUCB2 mRNA in peripheral tissues including pancreas, islets, heart, stomach, and also spinal cord (10, 20).

Adipocytes have been shown to produce a large number of peptides. However, few of them have been reported to possess true endocrine potencies or be regulated during metabolic perturbations and/or changes in nutritional status. Many studies associated these adipokines with a number of disease states including type 2 diabetes and obesity.

In our present study, we report novel findings demonstrating both the expression of NUCB2 mRNA and nesfatin-1 protein in both human and mouse adipose tissue and importantly its regulation by cytokines, insulin, and...
FIG. 5. Dose-dependent effects of TNF-α, IL-6, insulin, and dexamethasone on nesfatin-1 protein expression in 3T3-L1 adipocytes and sc adipose tissue explants and nesfatin-1 secretion from sc adipose tissue explants. Intracellular protein expression was determined by Western blot analysis and secretion was determined by ELISA. 3T3-L1 adipocytes (A–D) and sc adipose tissue explants (E–G) were cultured with or without TNF-α, IL-6, insulin, and dexamethasone for 24 h at indicated concentrations. For Western blot analysis, data were normalized against β-actin and were expressed as fold increase over basal. TNF-α, IL-6, insulin, and dexamethasone significantly increased nesfatin-1 protein expression in both 3T3-L1 cells and sc adipose tissue explants. IL-6, insulin, and dexamethasone significantly increased, and TNF-α significantly decreased, nesfatin-1 secretion in sc adipose tissue explants. All data are expressed as means ± SEM fold increase over control values. Ins, Insulin; Dex, dexamethasone. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered as significant compared with control group (n = 4).
corticosteroids in 3T3-L1 cells and adipose tissue explants. Moreover, we demonstrate that the secretion of nesfatin-1 into culture media from mouse sc adipose tissue explants and 3T3-L1 cells is influenced by metabolic regulators important in energy balance, suggesting that nesfatin-1 is a novel adipokine. Furthermore, circulating and adipose tissue protein levels of nesfatin-1 were higher in diet-induced obese mice. More importantly, human plasma nesfatin-1 positively correlated with increasing BMI.

A number of adipokines are produced in a site-specific manner. For example, leptin is produced predominantly by sc adipose tissue (21, 22), whereas visceral adipose tissue is the major source of OM (23). This general rule appears to hold true with nesfatin-1 because we found it to be predominantly expressed and secreted from sc compared with visceral adipose tissue. Similar findings were also observed for nesfatin-3, with higher protein levels in sc compared with OM adipose tissue (see Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). In addition, as is the case with leptin, NUCB2 gene expression was higher in adipocytes compared with stromal-vascular cells, as one might expect from an adipokine. The relevance of this higher expression in sc tissue, and indeed the site-specific expression of NUCB2, is uncertain at present but may suggest that NUCB2 may have an important role in determining functional properties particular to individual adipose depots.

Our findings show that the expression of nesfatin-1 was up-regulated in sc adipose tissue of mice fed a high-fat diet. This suggests a potential role for nesfatin-1 in the lipid accumulation pathway and perhaps diet-induced obesity. It is well known that leptin, adipokine predominantly secreted by sc adipose tissue, is increased in obese states and that fasting reduces leptin expression in both rodents and humans (24). Interestingly, we noted that food deprivation reduced the expression of nesfatin-1 in murine sc adipose tissue. Whether this occurs as a result of a decrease in the abundance of a population of adipocytes that express NUCB2, as has been described for leptin (25), is yet to be determined. Given that this is beyond the aim and scope of the current study, it would, however, be of interest to investigate this hypothesis.

The process of differentiation and maturation of adipocytes results in the increased expression of biologically active adipokines, such as leptin, adiponectin, and apelin (25, 26). It was no surprise therefore to find that nesfatin-1 followed a similar pattern with significantly increased expression during the process of differentiation of preadipocyte 3T3-L1 cells into mature adipocytes. It is possible that NUCB2 and nesfatin-1 may play a role in adipocyte development and differentiation, although the fact that it is secreted into the medium in increasing amounts as the adipocyte matures suggests that the increased expression is a by-product of maturation rather than a cause. Among other work, future studies should involve short hairpin RNA to silence NUCB2 during differentiation to evaluate the role and importance of NUCB2/nesfatin-1 in adipocyte differentiation.

It has been established that the plasma concentration of inflammatory mediators, such as TNF-α and IL-6, are increased in the insulin-resistant states of obesity, type 2 diabetes, and the met-
abolic syndrome (27). Interestingly, many of these inflammatory cytokines are produced and secreted by adipocytes and influence adipokine production. This prompted us to investigate the role of cytokines and hormones on nesfatin-1 expression in sc adipose tissue, particularly because the obese animals in our study had increased nesfatin-1 levels. Our findings demonstrate that TNF-α, IL-6, insulin, and dexamethasone increase the intracellular protein expression of nesfatin-1 in preadipocyte 3T3-L1 cells and mouse ex vivo sc adipose explants. However, the concentration of hormones/cytokines that induced maximal nesfatin-1 expression was slightly higher then the circulating levels. Under normal conditions circulating cytokines/hormones may have different effects on nesfatin-1 expression. Further studies using in particular in vivo models will hopefully address how these cytokines/hormones regulate nesfatin-1 production in adipose tissue. Furthermore, secretion of nesfatin-1 was measured in culture media from ex vivo explants, IL-6, insulin, and dexamethasone increased the secretion of nesfatin-1 comparable with intracellular protein expression. However, TNF-α showed a modest decrease in secretion of nesfatin-1 into culture media. These data are particularly interesting and suggest that the regulation of NUCB2 gene and nesfatin-1 intracellular protein production and the secretion may be mediated by different pathways. TNF-α has been shown to have differential effects on various adipokines. For example, IL-6, monocyte chemotactic protein-1, and metallothionein levels are increased after TNF stimulation, whereas adiponectin and leptin either decrease or show no significant change (28, 29).

Regulation of nesfatin-1 expression and secretion in adipocytes by insulin and other stimulus used in this current study may involve participation of key transcription factors such as PPARγ and trans-acting transcription factor 1 (Sp1) (30). Furthermore, insulin induces expression of PPARγ, in which PPARγ agonists have been shown to induce activation of NUCB2 a precursor of nesfatin-1 (5). This suggests PPARγ may be one of the transcription factors involved in insulin-mediated nesfatin-1 expression in adipocytes, but future work needs to address the possible involvement of other transcriptional factors, including Sp1.

In recent studies measuring plasma nesfatin-1 levels in healthy subjects, type 1 diabetes mellitus (T1 DM) and type 2 diabetes mellitus reported mean fasting nesfatin-1 levels to be slightly higher in T1 DM compared with type 2 diabetes mellitus patients. Furthermore, fasting plasma nesfatin-1 levels were significantly higher in T1 DM compared with healthy subjects. In addition, the same authors reported no sex-dependent changes in nesfatin-1 expression (31, 32). In agreement with these reports we also found no sex-dependent changes in plasma nesfatin-1 levels in our current study.

Nesfatin-1 is an anorexigenic factor that crosses the blood-brain barrier in both the blood-to-brain and brain-to-blood directions by a nonsaturable process (33). In addition, both central and peripheral administration of nesfatin-1 reduces food intake. However, what we noted is that obese animals have greater sc NUCB2/nesfatin-1 expression, as do humans with plasma nesfatin-1 levels positively correlating with increasing BMI. A similar observation is noted with leptin, another potent satiety molecule. Therefore, it would be of interest in future studies to investigate cerebrospinal fluid levels of nesfatin-1 in human subjects with varying BMIs.

In conclusion, the data presented in this study demonstrate that NUCB2/nesfatin-1 is a novel depot-specific adipokine preferentially expressed in sc adipose tissue/adipocytes. Adipose tissue NUCB2/nesfatin-1 expression increases with obesity and is altered in states of feeding and starvation. We present robust evidence that the secretion of nesfatin-1 into culture media dramatically increased during differentiation of 3T3-L1 cells into mature adipocytes and also by insulin, dexamethasone, and IL-6. Finally, NUCB2/nesfatin-1 should be added to the growing list of adipocyte-secreted molecules that are altered in dysmetabolic states, and future work is needed to elucidate the role of nesfatin in adipose tissue biology.

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