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Recombinant Human FIZZ3/Resistin Stimulates Lipolysis in Cultured Human Adipocytes, Mouse Adipose Explants, and Normal Mice

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Human FIZZ3 (hFIZZ3) was identified as an ortholog of mouse resistin (mResistin), an adipocyte-specific secreted factor linked to insulin resistance in rodents. Unlike mResistin, hFIZZ3 is expressed in macrophages and monocytes, but is undetectable in adipose tissue. The profound macrophage infiltration of adipose that occurs during obesity suggests that hFIZZ3 may play an important role in adipocyte biology. Using a recombinant protein produced in *Escherichia coli*, we report here that chronic treatment of cultured human adipocytes with hFIZZ3 results in hypotropic cells with smaller lipid droplets. Recombinant hFIZZ3 facilitates preadipocyte proliferation and stimulates adipocyte triglyceride lipolysis, whereas recombinant mResistin inhibits adipocyte differentiation, with no detectable effect on proliferation or lipolysis. In addition, insulin-stimulated glucose uptake and Akt phosphorylation are not altered in hFIZZ3-treated adipocytes, indicating an intact insulin response. In mouse adipose explants, hFIZZ3 accelerates simultaneously triglyceride lipolysis and fatty acid reesterification, as assessed by measurement of glycerol and fatty acid release. Consistent with the *in vitro* findings, acute administration of recombinant hFIZZ3 into normal mice caused a significant increase in serum glycerol concentration with no elevation in free fatty acid at 45 min post injection. Taken together, the data suggest that recombinant hFIZZ3 can influence adipose metabolism by regulating preadipocyte cell number, adipocyte lipid content, and energy expenditure via accelerating the fatty acid/triglyceride futile cycle. (*Endocrinology* 146: 2200–2209, 2005)

FIZZ3 BELONGS TO a FIZZ family of proteins that contain a characteristic C-terminal stretch of 10 cysteine residues with conserved spacing. To date, four members of the FIZZ family have been identified in mice, whereas only two members have been identified in humans. Human FIZZ3 (hFIZZ3) was cloned as an ortholog of the mouse FIZZ3; later named resistin (1). We will hereafter refer to human and mouse FIZZ3 as hFIZZ3 and mResistin, respectively, for clarification.

mResistin is an adipose-specific secreted factor associated with the development of insulin resistance in rodents (2). Gene expression of mResistin was induced during adipocyte differentiation and was regulated by hormones, nutritional status, and thiazolidinediones, a class of insulin sensitizers (2, 3). Treatment of normal mice with recombinant mResistin decreased glucose tolerance, and administration of anti-mResistin antibody improved insulin sensitivity and hyperglycemia in diet-induced obese mice (2). Initial studies proposed that mResistin led to insulin intolerance by inhibiting glucose uptake in adipose tissue; others showed that mResistin negatively affected insulin-stimulated glucose uptake in skeletal muscle, and recent reports emphasized mResistin-mediated impairment of hepatic insulin sensitivity (4–7). mResistin-deficient mice exhibited mild reduction of hepatic glucose synthesis without affecting muscle or adipose functions (6). On the contrary, transgenic mice overexpressing a dominant negative mResistin showed increased adiposity and improved insulin sensitivity due to enhancement of adipose differentiation, with no change in gluconeogenesis (8). Finally, Satoh and colleagues (9) demonstrated that adenovirus-mediated hyperresistinemia negatively affected insulin signaling in adipose tissue, skeletal muscle, and liver. Thus, although many reports agree on the causative role of mResistin in the development of insulin resistance in rodents, there is considerable disagreement regarding its proposed mechanism of action.

hFIZZ3 is 53% homologous to mResistin. The physiological function of hFIZZ3 and its role in development of insulin resistance remain unclear. Inconsistency in the correlation between serum hFIZZ3 levels and obesity or diabetes in patients complicates the picture. A positive correlation between the serum hFIZZ3 level and the patient's body mass index was reported in several studies (10–12), but not in others (13–15). In contrast to the adipose-specific distribution of mResistin, hFIZZ3 was expressed most abundantly in bone marrow (16). Analysis of hFIZZ3 expression in muscle and adipose biopsies from 42 individuals confirmed that hFIZZ3 was not expressed in those tissues (14). Similar results were reported by Fain *et al.* (17). An elegant study by Savage and colleagues (18) showed that hFIZZ3 expression in human adipose could not be attributed to adipocytes, preadipocytes, endothelial cells, or smooth muscle cells and could be detected exclusively in mononuclear cells and mac-

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Abbreviations: bFGF, Basic fibroblastic growth factor; BrdU, 5-bromo-2′-deoxyuridine; CT, threshold cycle; EthD-1, ethidium homodimer-1; FBS, fetal bovine serum; FFA, free fatty acid; IBMX, isobutylmethylxanthine; KRB, Krebs-Ringer bicarbonate buffer; -P, phosphorylated; RTQ-PCR, real-time quantitative PCR.

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rophages. A growing body of evidence suggested that obesity-induced adipose infiltration by macrophages and the resulting chronic inflammation might play critical roles in regulating adipose biology and obesity-associated metabolic disorders (19, 20). Expression of hFIZZ3 in monocytes and macrophages and its induction in response to proinflammatory agents made hFIZZ3 a good candidate for affecting adipose function (21). To our knowledge there was no report describing the potential role of hFIZZ3 in adipocyte biology. The goal of this study was to explore the effects of recombinant hFIZZ3 on adipocyte functions.

Materials and Methods

Materials

Recombinant hFIZZ3 and mResistin protein expressed in *Escherichia coli* were purchased from PeproTech (Rocky Hill, NJ). The concentrations of hFIZZ3 and mResistin were calculated based on their molecular masses of 19.5 and 20 kDa, respectively. Cryopreserved human sc preadipocytes were purchased from Zen-Bio, Inc. (Research Triangle Park, NC). DMEM/Ham's F-12 Nutrient Broth (1:1, vol/vol; DMEM/F12), Versene, antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin), fetal bovine serum (FBS), and rabbit serum were obtained from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Human epidermal growth factor, hTGF-β, human basic fibroblastic growth factor (bFGF), and recombinant human insulin were purchased from Roche (Indianapolis, IN). Krebs-Ringer bicarbonate buffer (KRB), biotin, pantothenate, isoproterenol, isobutylmethylxanthine (IBMX), dexamethasone, glycerol GPO Trinder reagents, 2-deoxy-D-glucose-1,2-^{3}H, nonradioactive 2-deoxy-D-glucose, cytochalasin B, and CL316–243 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Troglitazone was purchased from Biomol Research Laboratory (Plymouth Meeting, PA). F-moc-L-leucine and fatty acid-free BSA were obtained from Calbiochem (San Diego, CA). Bodipy (4,4-difluoro-3a,4a-diaza-s-indacene) fluorophore, and ethidium homodimer-1 (EthD-1) were purchased from Molecular Probes (Eugene, OR). Antihuman FIZZ3 antibody was purchased from PeproTech. Anti-Akt and antiphosphorylated Akt (anti-Akt-P) were obtained from Cell Signaling (Beverly, MA); anti-ERK1/2 and antiphosphorylated ERK1/2 (ERK1/2-P) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxidase-linked antirabbit IgG and the Enhanced Chemiluminescence Kit were obtained from Amersham Biosciences (Little Chalfont, UK). Mouse adipose tissues were obtained from 9-wk-old C57B/6J male mice (The Jackson Laboratory, West Sacramento, CA).

Quantitative RT-PCR

The expression of human FIZZ3 was quantified on microtiter plates containing RNA samples from human tissues using real-time quantitative PCR (RTQ-PCR). Total RNA from various human tissues was acquired from Stratagene (La Jolla, CA) and BioChain Institute (Hayward, CA). All tissue samples were received from certified hospitals with informed consent from donors or their legal representatives. RTQ-PCR was performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The RNA integrity of all samples was controlled for quality by visual assessment of agarose gel electropherograms using the 28S to 18S ribosomal RNA staining intensity ratio as a guide (2:1 to 2.5:1 for 28S:18S) and the absence of low molecular weight RNAs that would be indicative of degradation products. Samples were controlled against genomic DNA contamination by RTQ-PCRs run in the absence of reverse transcriptase using probe and primer sets designed to amplify a single exon. RNA samples were normalized to four reference genes. Normalized RNA (5 μl) was converted to cDNA and analyzed by RTQ-PCR using One Step RT-PCR Master Mix Reagents (Applied Biosystems) and gene-specific primers according to the manufacturer's instructions. PCRs were set up using TaqMan One-Step RT-PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. Reverse transcription was performed at 48 C for 30 min, followed by PCR amplification. After an initiation at 95 C for10 min, 40 cycles of amplification at 95 C for 15 sec and 60 C for 1 min were performed. Results were recorded as the threshold cycle (CT) value, which was the cycle at which a given sample crossed a threshold level of fluorescence; hence, a larger CT value represented a less abundant transcript (22). Transcripts with a CT higher the 35 were considered barely to undetectable in a given tissue. The percent relative expression was expressed as the percent difference in RNA concentration between a given sample and the sample with the lowest CT value. The relative expression was represented as 2 to the power of δ CT using a log scale. Probes and primers were designed according to the Applied Biosystems Primer Express software package (version I for Apple's Macintosh Power PC, Apple Computer, Cupertino, CA) using the human FIZZ3 gene sequence. The primer sequences used were: forward, 5′-CTGCAGAATGAAAGCTCTCTGT-3′; probe, 6-carboxyfluorescein-5′-CTCCTCCTCCCTGTCCTGGG-3′-tetramethylrhodamine; and reverse, 5′-GAGCACAGGGTCTTGCTAGA-3′.

Preparation of mouse preadipocytes

Primary mouse preadipocytes were isolated from sc adipose tissue according to a previously described method (22, 23). The isolated cells were plated in DMEM/F12 supplemented with 10% FBS and antibiotics. Medium was changed every other day. These cells display uniform fibroblastic morphology. For the proliferation assay, preadipocytes were used at passages 2–3.

Human adipocyte differentiation

Frozen human preadipocytes were thawed and cultured in preadipocyte expansion medium (DMEM/F12 supplemented with 10% FBS, human epidermal growth factor (2.5 ng/ml), hTGF-β (0.25 ng/ml), bFGF (0.5 ng/ml), and antibiotics until the cells were confluent. For adipocyte terminal differentiation, preadipocytes were induced with differentiation medium containing DMEM/F12, 3% rabbit serum, biotin (33 μM), pantothenate (17 μM), human recombinant insulin (0.1 μM), dexamethasone (1 μM), IBMX (0.5 mM), and a peroxisome proliferator-activated receptor γ ligand, such as troglitazone (3 μM) or F-moc-L-leucine (30 μM) (24). After a 6-d induction period, with fresh medium added every 3 d, the cells were fed with the same medium without IBMX or a peroxisome proliferator-activated receptor γ ligand (adipocyte medium) every 3–4 d for an additional 6 d. Cells at passages 3–4 were routinely used for adipocyte differentiation. Lipid accumulation was confirmed by staining formaldehyde-fixed cells with the neutral lipid-specific dye Bodipy at 1 μg/ml as previously described (25). Microscopic images were obtained using an Axiovert 200M inverted microscope with NHBO103 Illuminator and AxioCam MRc Image Acquisition System (Carl Zeiss, Inc., Munich, Germany).

Adipocyte lipid-DNA ratio

Differentiated adipocytes with or without hFIZZ3 treatment were fixed in formaldehyde (5%), and triglyceride was stained with Bodipy as described above. After the fluorescence of each well was read at 530 nm, the Bodipy-stained lipid was removed, and cells were permeabilized using isopropanol. Isopropanol was subsequently removed, and cellular DNA was stained with EthD-1 (2 μM) for 30 min before measuring EthD-1 fluorescence at 620 nm (26). The EthD-1 readout is directly correlated with cell density between 2,000 and 40,000 cells/cm^2. Fluorescence units of Bodipy and EthD-1 per well were used to determine the lipid/DNA ratio.

Cell proliferation

Preadipocytes were plated at a density of 5000 cells/cm^2 in preadipocyte medium (DMEM/F12 with 10% FBS) overnight to allow adherence. Subsequently, medium was changed to DMEM/F12 with 5% FBS and the indicated concentrations of recombinant hFIZZ3 or mResistin. After 24 h of incubation at 37 C with 5% CO_2, cells were pulsed for another 24 h with 5-bromo-2′-deoxyuridine (BrdU). BrdU incorporation was quantified by ELISA using the Cell Proliferation ELISA kit following the manufacturer's instructions (Roche). bFGF at 2 ng/ml was used as a positive control. To determine the molecular mechanism of hFIZZ3-induced proliferation, human preadipocytes in DMEM/F12 supplemented with 5% FBS were treated with vehicle or 1 μM hFIZZ3 for 15 min. Equal amounts of protein (10 μg) were analyzed by Western blot-

ting with anti-ERK1/2-P and anti-Akt-P antibodies (1:1000) to assess the cellular response to hFIZZ3 or with anti-ERK1/2 and anti-Akt antibody (1:1000) to determine the amount of total (phosphorylated and nonphosphorylated) protein in control and treated cells.

Lipolysis in vitro

To compare the effects of hFIZZ3 and mResistin, human adipocytes in 96-well plates were incubated in KRB containing 0.5% fatty acid-free BSA (KRB-BSA) with the indicated concentrations of hFIZZ3 or mResistin for 5 h, and glycerol release was measured using reagents in the GPO-Trinder kit. IBMX at 0.1 mM was used as a positive control. To show that depletion of hFIZZ3 protein abolished the observed lipolytic activity, 1 ml hFIZZ3 (1 μM) was immunoprecipitated with 20 μl anti-hFIZZ3 antibody bound to protein G beads at 4 C overnight. After centrifugation to remove protein-antibody complexes attached to protein G beads, the supernatant was used as the stock solution to prepare various concentrations for lipolysis assay. To assess lipolysis in adipose explants, mouse fat pads were minced and weighed. Approximately 100 mg adipose were placed in a microtube containing 500 μl KRB-BSA with vehicle, recombinant protein, or a control. Aliquots were removed at 0 and 5 h. Glycerol released was measured as described above. Fatty acids in conditioned medium were measured using a free fatty acid (FFA) colorimetric assay (Roche). Glycerol and fatty acid release was normalized by tissue weight.

Lipolysis in vivo

Male 8-wk-old C57BL/6J mice (The Jackson Laboratory) were maintained in a 12-h light, 12-h dark cycle with food (LabDiet no. 5P75 irradiated; 22% protein, 13% fat, and 60% carbohydrates; PMI Nutrition International, St. Louis, Mo) and water provided *ad libitum*. All procedures were conducted based on protocols approved by The Jackson Laboratory institutional animal care and use committee. The study was conducted with guidelines presented by the Guide for the Care and Use of Laboratory Animals. After a 7-d acclimation period, the animals were weighed and randomly allocated to the various treatment groups (n = 5/treatment group). Animals then received an ip injection of the hFIZZ3 (0.1, 2, and 20 mg/kg) or CL316–243 (3 mg/kg), and groups of mice were then killed at 45, 180, and 360 min post injection. Compounds were administered 4 h after the start of the light cycle. The dose of both agents administered was corrected to body weight. Terminal blood samples were collected via cardiac puncture and centrifuged (2000 rpm; 10 min; 4 C), and serum was stored frozen at −80 C until analysis. Serum glycerol and fatty acid levels were determined as described above. The serum insulin level was measured using the mouse insulin ELISA kit (Alpco Diagnostics, Windham, NH). The serum hFIZZ3 level was assessed using a human FIZZ3 ELISA following the manufacturer's instructions (PeproTech).

Western blot analysis of insulin-stimulated Akt phosphorylation

Differentiated human adipocytes were rested in serum-free DMEM containing 0.15% BSA overnight. Cells were then treated with or without hFIZZ3 protein for the indicated time periods, stimulated with 0 or 10 nM insulin for 15 min, and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100 supplemented with phosphatase and protease inhibitors. The protein content was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of protein were resolved by SDS-PAGE, followed by transfer to a nitrocellulose membrane. The membranes were probed with anti-Akt-P antibody (1:1000) to assess cellular insulin response or with an anti-Akt antibody (1:1000) to determine total Akt protein. Detection was performed using an enhanced chemiluminescence method with peroxidase-linked antirabbit IgG (1:5000) according to the manufacturer's instructions.

Insulin-stimulated glucose uptake

Differentiated human adipocytes in 96-well plates were rested overnight as described above. The indicated concentrations of hFIZZ3 were added for 5 and 18 h before insulin stimulation. To measure insulin-mediated glucose uptake, insulin was added to a final concentration of 10 nM for 30 min before the addition of 1.0 μCi/ml 2-deoxy-D-glucose-1,2-^{3}H for a 2-h incubation. Subsequently, cells were washed twice with ice-cold PBS and lysed with 0.2 N NaOH. Nonspecific glucose uptake was determined in the presence of 10 μM cytochalasin B with insulin. Glucose uptake was determined using an I450 Microbeta Trilux liquid scintillation counter (PerkinElmer, Norwalk, CT).

Statistical analysis

Cellular assay data were analyzed by ANOVA. Data fulfilled all assumptions for parametric analysis. Significance was set at the 0.05 level. If significant differences were observed, means were separated using Dunnett's test.

Results

Expression of hFIZZ3

The expression of hFIZZ3 from various adult and fetal tissues was examined by quantitative RTQ-PCR. The 73-bp amplicon corresponded to the sequence in exon 2 of the hFIZZ3 gene that contained the first 49 bp of coding region. The expression of both full-length hFIZZ3 and a recently described alternatively spliced isoform that lacked of entire exon 3 (27) was assessed using this amplicon. hFIZZ3 mRNA was predominantly expressed in bone marrow (the source of macrophages and monocytes), lung, and several fetal tissues (Table 1). There was no detectable hFIZZ3 expression in human preadipocytes, human adipocytes (data not shown), or human adipose tissue.

TABLE 1. Expression of hFIZZ3 across the panel of human tissues by RTQ-PCR

Human tissue	CT value	Relative expression, (%)
Bone marrow	27.6	100.0
Liver (fetal)	29.3	30.6
Lung (fetal)	30.1	17.9
Lung	30.4	14.2
Heart (fetal)	31.1	8.8
Kidney (fetal)	33.0	2.3
Thymus	33.3	1.9
Spleen	33.6	1.6
Fetal Skeletal	33.8	1.3
Uterus	34.2	1.0
Placenta	34.4	0.9
Spinal cord	34.8	0.7
Skeletal muscle	34.9	0.6
Trachea	35.1	0.5
Brain (fetal)	35.5	0.4
Small intestine	35.7	0.4
Ovary	36.0	0.3
Bladder	36.2	0.3
Adrenal gland	36.4	0.2
Colorectal	37.0	0.1
Stomach	37.6	0.1
Thyroid	38.2	0.1
Pituitary gland	38.7	0
Lymph node	39.5	0
Heart	39.6	0
Kidney	39.7	0
Adipose	40	0
Brain (whole)	40	0
Liver	40	0
Mammary gland	40	0
Pancreas	40	0
Prostate	40	0
Salivary gland	40	0
Testis	40	0

Results are expressed as CT values and as a percentage of relative expression compared to the tissue with the highest expression. CT values of 35 and higher are considered a background level.

Protein characterization

hFIZZ3 has been shown to dimerize through a disulfide bond formed by the N-terminal cysteine (Cys^{21}), and formation of an oligomeric structure was proposed to be important to its function (25, 26). Purified recombinant hFIZZ3 produced in *E. coli* appeared as a dimer with molecular mass of 19.5 kDa on a nonreducing SDS-PAGE and a mixture of dimer and monomer on reducing SDS-PAGE with molecular weights of 19.5 and 10 kDa, respectively. There were no additional protein bands visible with Coomassie blue R-250 staining. Matrix-assisted laser desorption ionization-time of flight analysis of the recombinant protein showed a single peak corresponding to the dimeric form of hFIZZ3 with no detectable contamination (data not shown). Similarly, mResistin appeared as a dimer of 20 kDa on a nonreducing SDS-PAGE.

Effect of hFIZZ3 on human adipocyte differentiation

It was reported that mResistin attenuated differentiation of mouse adipocytes *in vitro* (3). To assess whether hFIZZ3 exhibited similar activity, we determined the effect of recombinant hFIZZ3 on the differentiation of human adipocytes.

Human sc preadipocytes were treated with recombinant hFIZZ3 or mResistin for 9 d during the period of preadipocyte expansion and initiation of differentiation. Cells were cultured for an additional 6 d in adipocyte medium without recombinant protein to allow continued lipid accumulation. Intracellular lipids were visualized by staining the cells with Bodipy at the end of the 12-d period. Microscopic examination of hFIZZ3-treated cells showed an increase in the number of cells per field with smaller lipid droplets in each cell (Fig. 1A). To quantify the observed morphological changes, the lipid/DNA ratio was determined by double staining the cells with the lipid-specific dye Bodipy and the nucleic acid-specific dye EthD-1. In agreement with the microscopic evaluation, Bodipy fluorescence per well was decreased, whereas EthD-1 fluorescence per well was increased in response to hFIZZ3 treatment. As a result, the lipid/DNA ratio in hFIZZ3-treated cells was dose-dependently reduced ($P < 0.0001$; Fig. 1B). TNFα, an inhibitor of adipocyte differentiation, suppressed lipid accumulation with no detectable Bodipy staining in response to a 9-d treatment at 10 ng/ml (Fig. 1, A and C). mResistin-treated cells had reduced cellular lipid content compared with TNFα-treated cells without an affect on cell number (Fig. 1C).

Effects of hFIZZ3 and mResistin on preadipocyte proliferation

To confirm that hFIZZ3 increased cell number, as observed from microscopic analysis, human preadipocyte proliferation was determined via BrdU incorporation. As shown in Fig. 2A, treatment of human preadipocytes with hFIZZ3

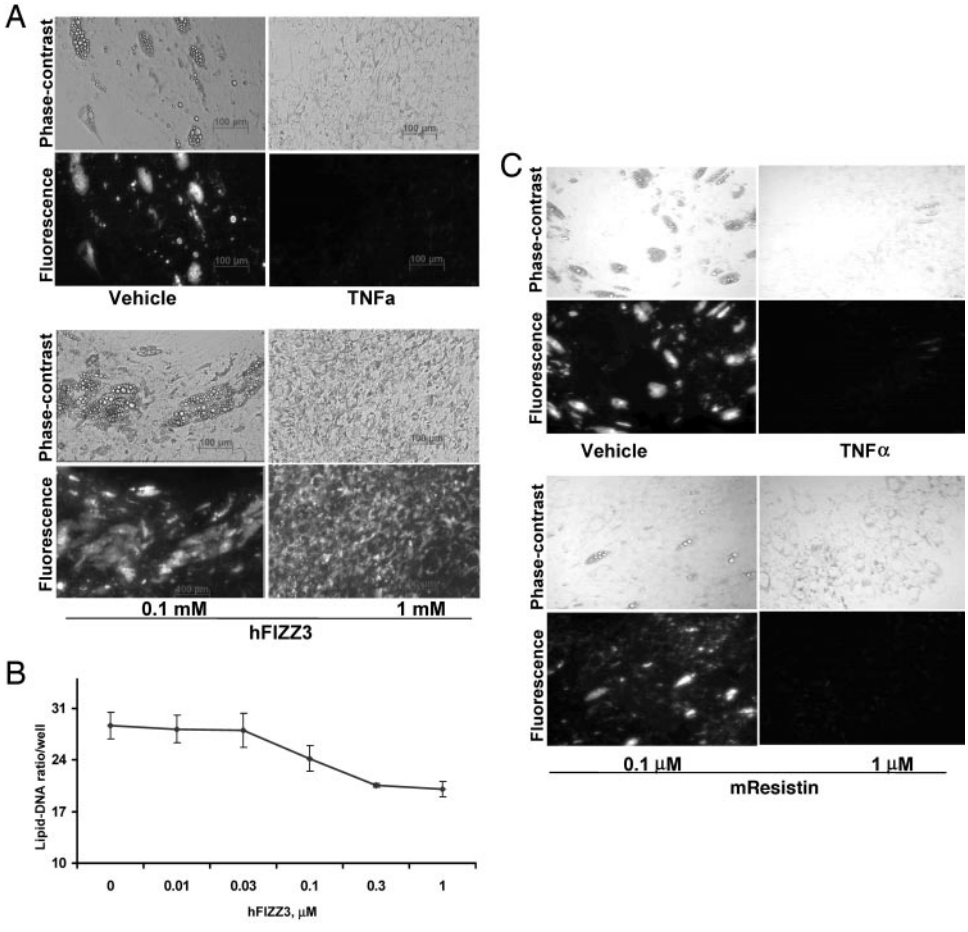

FIG. 1. hFIZZ3 increased adipocyte cell number and decreased the size of cellular lipid droplets. A and C, Human preadipocytes were treated with the indicated concentrations of hFIZZ3 (A) or mResistin (C) for 9 d after plating during the period of preadipocyte expansion and differentiation, followed by an additional 6-d lipid accumulation period in adipocyte medium without addition of recombinant proteins. Adipocyte lipid accumulation was determined by staining with Bodipy as described in the text. The phase contrast pictures showed cell morphology. The fluorescence pictures show the Bodipy-stained, lipid-containing cells. TNF-α at 10 ng/ml was used as a control for monitoring inhibition of adipocyte differentiation. B, Lipid/DNA ratio of adipocytes treated as described above with the indicated concentrations of hFIZZ3 was determined by quantification of double staining for Bodipy and EthD-1.

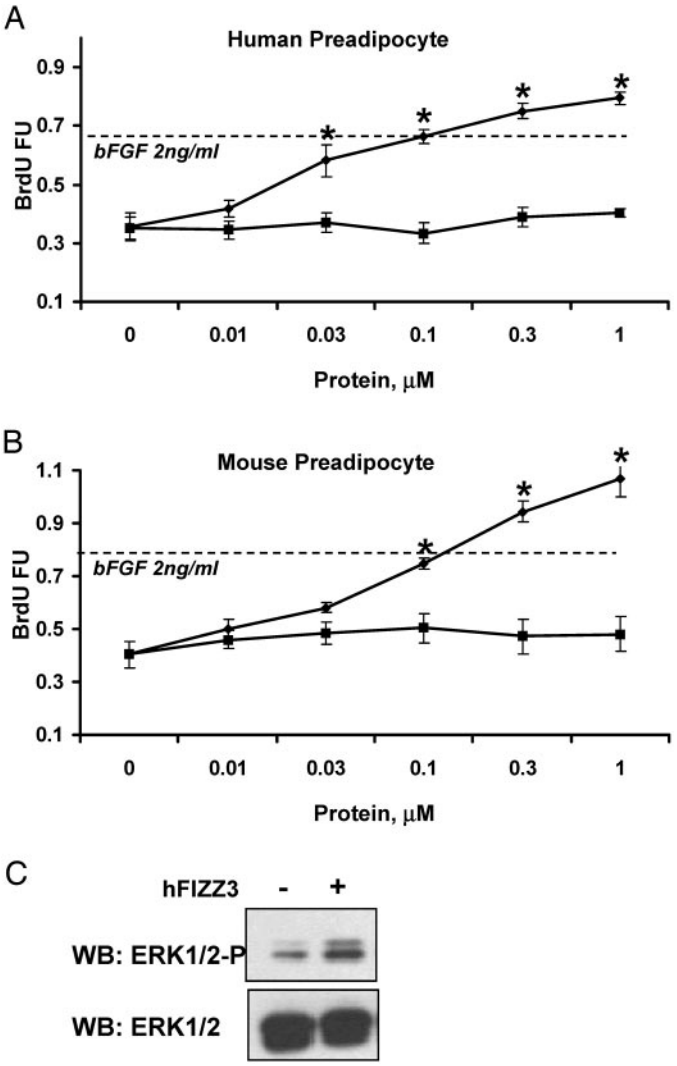

FIG. 2. hFIZZ3 induced preadipocyte proliferation. A and B, Preadipocytes were incubated in medium containing 5% FBS and the indicated concentrations of recombinant protein for 24 h. Cell proliferation was measured by BrdU incorporation for 24 h. hFIZZ3 (♦) and mResistin (■) were tested using human preadipocytes (A) or mouse preadipocytes (B). Results are expressed as the mean ± SD of triplicate measurements and are representative of three independent experiments. *, By Dunnett's test between BrdU absorbance in hFIZZ3-treated (each point of the curve) *vs.* vehicle-treated cells, $P < 0.05$. C, Human preadipocytes in medium containing 5% FBS were treated with vehicle or 1 μM hFIZZ3 for 15 min, and cell lysates were collected. Ten micrograms of protein were loaded on a sodium dodecyl sulfate gel, followed by Western blotting with anti-phospho-ERK1/2 antibody. To confirm that equal amounts of ERK1/2 protein were loaded, a Western blot with anti-ERK1/2 antibody was performed.

caused a statistically significant and dose-dependent increase in preadipocyte proliferation ($P < 0.0001$). mResistin did not influence human preadipocyte proliferation at any of the tested concentrations. To assess whether hFIZZ3 and mResistin had the same activity in mouse cells, similar experiments were conducted using primary preadipocytes isolated from mouse sc fat pads (Fig. 2B). hFIZZ3, but not mResistin, led to a significant induction of BrdU incorporation in mouse preadipocytes ($P < 0.0001$). The proliferation of human lung carcinoma cell lines H522, H1299, and H460 or mouse NIH-3T3 cells (data not shown) did not change in response to hFIZZ3 treatment suggesting some specificity of the observed proliferative activity of hFIZZ3. Recently, it was shown that recombinant hFIZZ3 promoted smooth muscle cell proliferation via activation of ERK1/2 and phosphotidylinositol 3-kinase (PI3K) signaling pathways (28). To better understand the molecular mechanisms involved in hFIZZ3-induced preadipocytes proliferation, we investigated the phosphorylation of ERK1/2 and Akt kinase in human preadipocytes treated with hFIZZ3. As shown in Fig. 2C, hFIZZ3 increased ERK1/2 phosphorylation, which indicated involvement of MAPK signaling pathway in hFIZZ3-mediated proliferation. There was a low level of Akt phosphorylation in vehicle-treated human preadipocytes that was not changed by hFIZZ3 addition (data not shown).

Effect of hFIZZ3 on lipolysis in vitro

To understand the observed decrease in lipid droplet size in human adipocytes, we investigated the effects of hFIZZ3 on lipolysis. Triglyceride hydrolysis or lipolysis leads to the release of both glycerol and fatty acids. Lipolysis in primary culture was assessed by glycerol release only because the level of FFAs in conditioned medium from cultured adipocytes was too low to be measured using a FFA kit. Human adipocytes treated with hFIZZ3 for various periods of time ranging from 2–24 h showed a time-dependent induction of lipolysis (data not shown). Treatment for 5 h was chosen for the subsequent experiments. As shown in Fig. 3A, hFIZZ3 dose-dependently increased glycerol release in cultured human adipocytes ($P < 0.0001$). In contrast, there was no change in lipolysis by mResistin (Fig. 3A). Using immunoprecipitation with an anti-hFIZZ3 antibody, we confirmed that the observed activity in the assay was indeed attributed to hFIZZ3. Immunoprecipitation resulted in almost complete removal of hFIZZ3 from the starting material, as shown by Western blotting analysis (Fig. 3B). Treating adipocytes with hFIZZ3-depleted supernatant did not cause any alteration in glycerol release (Fig. 3A).

To demonstrate that hFIZZ3 had similar lipolytic activity across species, we examined lipolysis in mouse adipocytes. In our hands, mouse preadipocytes did not differentiate sufficiently *in vitro* to allow accurate measurement of glycerol release. Therefore, we examined lipolysis in mouse adipose explants. Glycerol and fatty acid release was determined in the conditioned medium from approximately 100 mg mouse adipose tissue. hFIZZ3 dose-dependently promoted glycerol release in mouse adipose tissue ($P < 0.0001$), whereas it did not significantly change fatty acid release ($P = 0.065$; Fig. 4). The amount of fatty acid in conditioned medium is regulated by equilibrium between fatty acid release from lipolysis and uptake for reesterification back to triglyceride. Hydrolysis of one molecule of triglyceride will release one molecule of glycerol and three molecules of FFA, thus yielding a molar ratio of FFA/glycerol of 3:1 if no significant reesterification takes place. As expected, the molar ratio of FFA/glycerol in vehicle-treated mouse adipose tissue was 2.7 and did not change significantly in IBMX- or isoproterenol-treated explants. In contrast, hFIZZ3 dose-dependently decreased the molar ratio of FFA/glycerol from 2.6 to 1.8 at the maximal

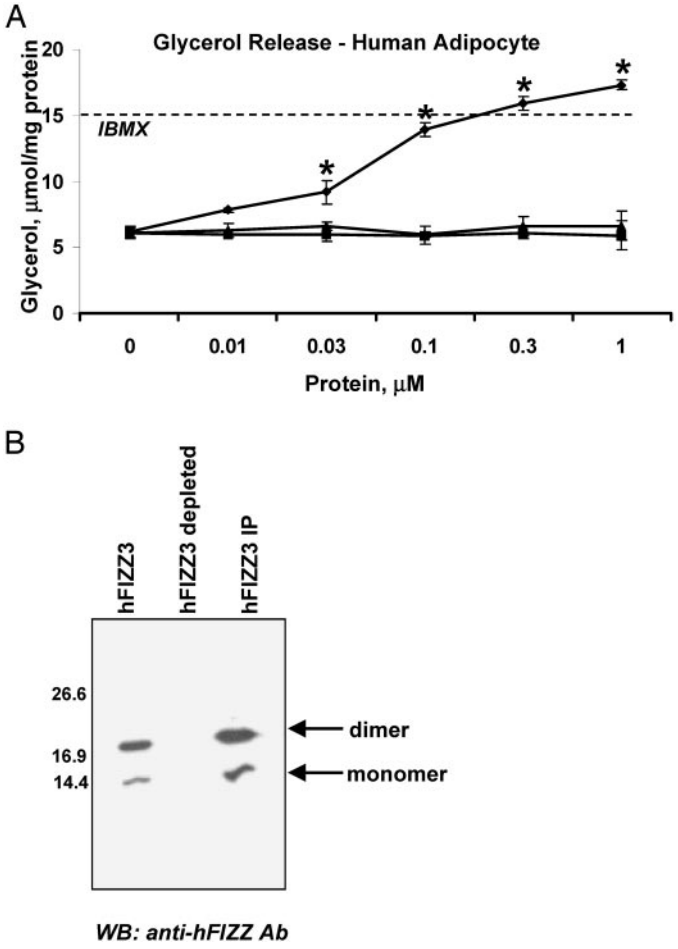

FIG. 3. hFIZZ3 stimulated lipolysis in cultured human adipocytes. Human adipocytes were incubated in KRB-BSA with or without the indicated amounts of hFIZZ3 (♦), depleted hFIZZ3 (▲), mResistin (■), or control (IBMX at 10^{-4} M or isoproterenol at 10^{-5} M) for 5 h. An aliquot of conditioned medium was collected, and glycerol released was measured as described in *Materials and Methods*. Results are expressed as the mean ± SD of triplicate measurements and are representative of three independent experiments. *, By Dunnett's test between glycerol release in hFIZZ3-treated (each point of the curve) *vs.* vehicle-treated cells, $P < 0.05$. B, Depletion of hFIZZ3 by immunoprecipitation was confirmed by Western blot analysis.

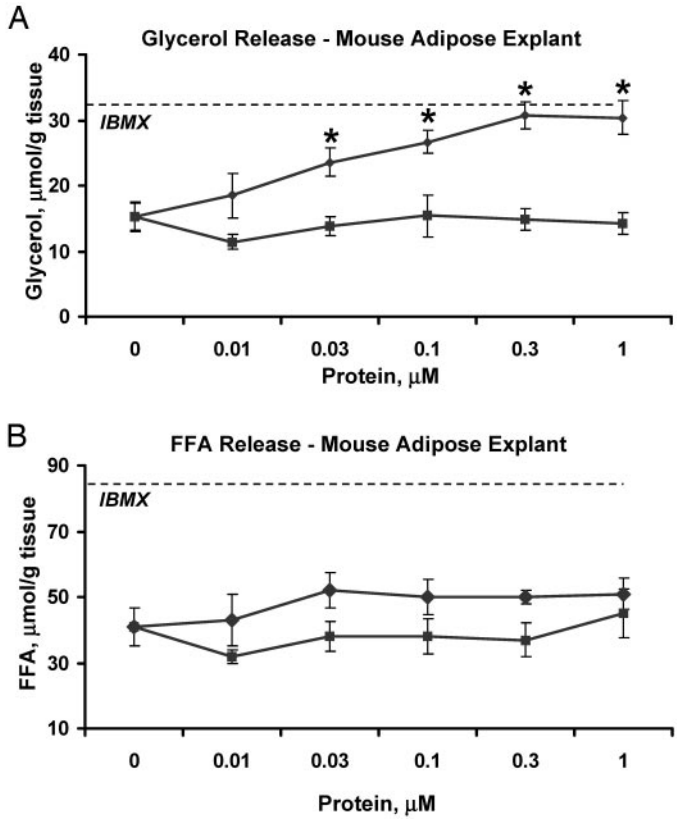

FIG. 4. hFIZZ3 stimulated triglyceride adipocyte and fatty acid re-esterification in mouse adipose explants *ex vivo*. Mouse adipose explants were incubated in KRB-BSA with or without the indicated amounts of hFIZZ3 (♦), mResistin (■), or control (IBMX at 10^{-4} M or isoproterenol at 10^{-5} M) for 5 h. Glycerol (A) and FFA (B) released in conditioned medium were measured as described in *Materials and Methods*. Results are expressed as the mean ± SD of triplicate measurements and are representative of two independent experiments. *, By Dunnett's test between glycerol release in hFIZZ3-treated (each point of the curve) *vs.* vehicle-treated adipose explants, $P < 0.05$.

concentration of hFIZZ3 ($P = 0.001$). In a parallel experiment, mResistin did not alter glycerol or fatty acid release in mouse adipose explants (Fig. 4).

Effect of hFIZZ3 on lipolysis in vivo

To determine whether hFIZZ3 administration also induces lipolysis *in vivo*, C57BL/6J mice received a single ip injection of recombinant hFIZZ3 (0.1, 2, and 20 mg/kg) or CL316-243 (3 mg/kg), a β_3-adrenergic receptor agonist. Blood samples were collected at 45, 180, and 360 min after injection, and serum glycerol, FFA, glucose, insulin, and recombinant hFIZZ3 levels were measured (Table 2). Sampling times were selected based on the reported lipolytic effect of CL-316-243 assessed via an increase in plasma glycerol levels (29), because there are no available data regarding the kinetics of hFIZZ3 effect on glycerol level *in vivo*. Serum glycerol concentration in the CL316-243-treated group was significantly higher than the control group at 45 and 180 min after treatment ($P < 0.001$). A significant dose-dependent increase in serum glycerol concentration occurred in the hFIZZ3-treated group at 45 min ($P < 0.001$), whereas a decrease occurred at 360 min post treatment ($P < 0.001$). CL316-243-treated group had a significantly higher serum FFA concentration than the control group at 45 and 180 min post treatment ($P < 0.05$). No significant differences in serum FFA concentration were present between hFIZZ3 and control groups at 45 min. The serum FFA concentration exhibited a decreasing linear dose trend with hFIZZ3 treatment at 180 and 360 min post treatment ($P < 0.05$). The serum glucose concentration was significantly lower ($P < 0.05$), whereas insulin level was significantly higher ($P < 0.001$) in the CL316-243-treated group than control group at 45 and 180 min post treatment. hFIZZ3 administration had no significant effect on serum glucose or insulin concentrations. Serum levels of recombinant hFIZZ3 were monitored via ELISA. The increase in serum hFIZZ3 levels was a reflection of the dose administered. The anti-hFIZZ3 antibody used in the ELISA did not exhibit significant cross-reactivity with mResistin as no signal was detected in the ELISA as well as Western blot (data not shown).

TABLE 2. Effect of acute administration of hFIZZ3 (0.1, 2, and 20 mg/kg, ip) or CL316-243 (3 mg/kg, ip) to male C57BL/6J mice on serum glycerol, FFA, glucose, insulin, and hFIZZ3 concentrations

Time (min)	Treatment	Serum concentration				
		hFIZZ3 (ng/ml)	Glycerol (μM)	FFA (mM)	Glucose (mg/dl)	Insulin (ng/ml)
45	Vehicle	ND	158 ± 8	1.5 ± 0.7	283 ± 40	1.4 ± 0.2
	CL316-243	ND	451 ± 23[a]	4.8 ± 1.2[a]	201 ± 45[b]	10.9 ± 1.4[a]
	hFIZZ3 (0.1 mg/kg)	11 ± 3	197 ± 14[a]	2.4 ± 0.6	290 ± 42	1.5 ± 0.1
	hFIZZ3 (2 mg/kg)	36 ± 3	238 ± 10[a]	2.1 ± 0.5	295 ± 25	1.4 ± 0.2
	hFIZZ3 (20 mg/kg)	60 ± 10	341 ± 36[a]	2.2 ± 0.6	282 ± 33	1.3 ± 0.2
180	Vehicle	ND	166 ± 15	2.2 ± 0.7	255 ± 25	1.5 ± 0.1
	CL316-243	ND	348 ± 28[a]	3.4 ± 0.6[a]	172 ± 34[b]	5.6 ± 0.6[a]
	hFIZZ3 (0.1 mg/kg)	12 ± 3	215 ± 32	2.0 ± 0.7	271 ± 64	1.4 ± 0.1
	hFIZZ3 (2 mg/kg)	40 ± 9	252 ± 13	1.6 ± 0.4	292 ± 50	1.4 ± 0.4
	hFIZZ3 (20 mg/kg)	48 ± 8	152 ± 35	0.8 ± 0.4[b]	258 ± 93	1.4 ± 0.3
360	Vehicle	ND	178 ± 7	1.5 ± 0.9	244 ± 30	1.5 ± 0.4
	CL316-243	ND	188 ± 15	1.7 ± 1.4	215 ± 83	2.2 ± 1.1
	hFIZZ3 (0.1 μg/kg)	7 ± 2	137 ± 18[a]	0.7 ± 0.3	253 ± 40	1.3 ± 0.1
	hFIZZ3 (2 mg/kg)	13 ± 2	119 ± 27[a]	0.6 ± 0.2[b]	284 ± 25	1.4 ± 0.5
	hFIZZ3 (20 mg/kg)	33 ± 3	109 ± 11[a]	0.4 ± 0.09[b]	235 ± 40	1.3 ± 0.2

Animals received the compounds 4 h after the beginning of the light cycle. Mice were killed 45, 180, and 360 min after injection. ND, Not detected. Values in a *column* within a time point were compared with controls using Dunnett's test. Values shown are the mean ± SE (n = 5).
[a] $P < 0.001$.
[b] $P < 0.05$.

Effect of human FIZZ3 on insulin signaling and glucose uptake

Adipocyte lipid accumulation can also be regulated by glucose uptake, because glucose is the primary carbon source under these culture conditions. Exposure to insulin significantly increased glucose uptake up to 2-fold compared with nonstimulated human adipocytes ($P < 0.0001$). However, cells treated with hFIZZ3 displayed no dependence of insulin response with hFIZZ3 concentration ($P = 0.4050$) or with time of treatment ($P = 0.4482$; Fig. 5A). To further assess the effect of hFIZZ3 on insulin signaling, insulin-stimulated phosphorylation of Akt was measured 5 or 18 h after hFIZZ3 treatment. As shown in Fig. 5B, the addition of hFIZZ3 did not alter insulin-mediated Akt phosphorylation at either time point.

Discussion

Adipose is an important endocrine organ, secreting a number of factors called adipokines. Adipokines, the biologically active polypeptides produced exclusively or substantially by adipocytes, are involved in regulation of lipid metabolism and energy homeostasis. Among the adipokines, rodent resistin is linked to the development of glucose intolerance. Its human ortholog, referred to here as hFIZZ3, is not an adipokine, because it is expressed in macrophage and monocytes, but is undetectable in adipocytes. Differences in the sequences in intronic region between rodent and human FIZZ3 that contains many regulatory elements are likely to explain the difference in tissue distribution (16). The presence of hFIZZ3 in the cells of immune origin might account for the detection of hFIZZ3 mRNA and/or protein in adipose tissue reported by some researchers (11, 15). Infiltration of adipose tissue by macrophages suggests that hFIZZ3 might play an important role in adipocyte metabolism. Based on extensive research on its mouse ortholog, hFIZZ3 is thought to negatively affect insulin sensitivity in peripheral tissue, including adipose. However, moderate sequence homology in coding and noncoding regions, differences in tissue distribution, and overall poor conservation of FIZZ family across the species call for caution in extrapolating mResistin data to hFIZZ3. To explore the potential role of hFIZZ3 in adipose biology, we examined the effect of recombinant hFIZZ3 produced in *E. coli* on adipocyte differentiation, preadipocyte proliferation, lipolysis, and insulin signaling.

We report that recombinant hFIZZ3 has unexpected and interesting effects on adipocyte functions. Specifically, prolonged *in vitro* exposure to recombinant hFIZZ3 results in an adipocyte population with smaller cellular lipid droplets. Treatment with recombinant hFIZZ3 enhances proliferation in primary preadipocyte and glycerol release in mature adipocytes. Both induction of lipolysis and proliferation might explain the decrease in lipid droplet size upon chronic hFIZZ3 treatment *in vitro*. We also show that stimulation of human preadipocytes with recombinant hFIZZ3 leads to activation of the ERK1/2 signaling pathway, an important mediator of cell growth and proliferation. The data are in agreement with recent finding that hFIZZ3 increases smooth muscle cell proliferation via activation of the MAPK signaling pathway (28). The same signaling pathway is implicated in the regulation of lipolysis in mature adipocytes (30, 31). Whether the ERK1/2 signaling pathway is indeed involved in hFIZZ3-mediated induction of lipolysis remains a question for future investigation.

Measurement of glycerol and FFA output from mouse adipose explants shows that hFIZZ3 induces a greater degree of glycerol release than of fatty acids. The data suggest that recombinant hFIZZ3 activates both triglyceride lipolysis and fatty acid reutilization. In this regard, hFIZZ3 resembles leptin, which is known to accelerate both lipolysis and reesterification and to promote the adipose triglyceride/fatty acid

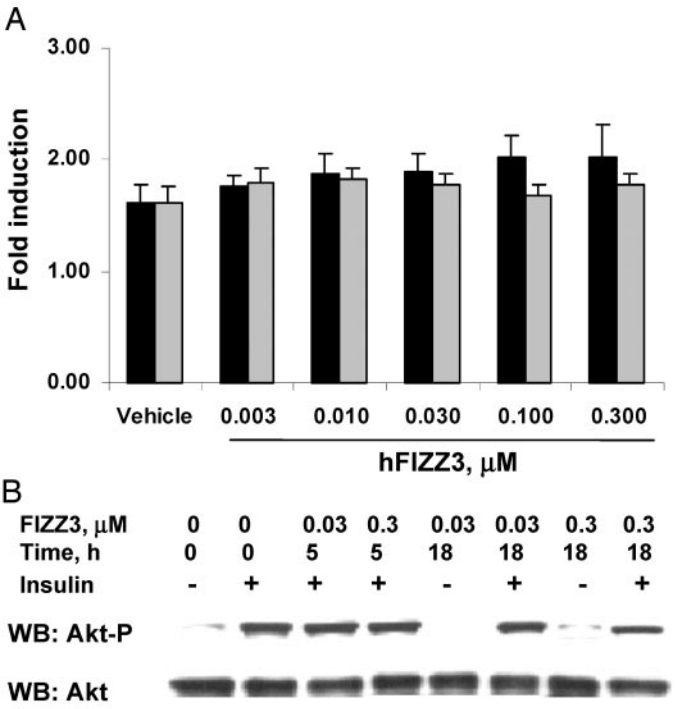

FIG. 5. hFIZZ3 did not affect insulin signaling in adipocytes. A, Effect of hFIZZ3 on insulin-stimulated glucose uptake in human adipocytes. Differentiated human adipocytes were rested in serum-free medium overnight. The indicated concentrations of hFIZZ3 were added 5 h (■) and 18 h (▒) before insulin stimulation. Cells were stimulated with 10 nM insulin for 30 min, and ^{3}H-labeled glucose was added to the medium. After a 2-h incubation, glucose uptake was measured as described in *Materials and Methods*. The results are representative of triplicate measurements from three independent experiments. B, Effect of hFIZZ3 on insulin-stimulated Akt phosphorylation in human adipocytes. Differentiated human adipocytes were rested in serum-free medium overnight. The indicated concentrations of hFIZZ3 were added to the medium 5 or 18 h before insulin stimulation. Adipocytes were stimulated with 10 nM insulin for 30 min, and cell lysates were collected. Thirty micrograms of protein were loaded on a sodium dodecyl sulfate gel, followed by Western blotting with anti-Akt-P antibody. To confirm that equal amount of Akt protein was loaded, a Western blot with anti-Akt antibody was performed.

substrate cycle and energy expenditure at high concentrations (32–34).

Acute administration of hFIZZ3 to normal mice increased the serum glycerol concentration maximally by 2.1-fold of the basal level 45 min post injection without a significant change in the FFA level. This finding confirmed the results of our *in vitro* experiments that recombinant hFIZZ3 activated both lipolysis and fatty acid reesterification. We did not detect an elevation of glycerol 180 and 360 min post injection in hFIZZ3-treated mice despite the significant serum level of recombinant hFIZZ3 protein measured by ELISA. However, the concentration of intact hFIZZ3 might be much less, because polyclonal antibody used in ELISA could also recognize various degradation fragments of hFIZZ3. Surprisingly, there is a significant drop in fatty acid levels at 180 and 360 min and in glycerol level at 360 min post injection in hFIZZ3-treated animals. Additional animal studies will need to be conducted to understand whether this reduction of glycerol and fatty acid levels might be explained by a rebound effect after an initial increase in lipolysis or by other physiological responses involving increased FFA and glycerol tissue uptake.

Recombinant hFIZZ3 did not alter insulin action in human adipocytes judging by the similar induction of glucose uptake and Akt phosphorylation upon insulin stimulation. Even though our results do not rule out the possibility that hFIZZ3 might affect insulin signaling in skeletal muscle or liver, the similar blood glucose and insulin levels in mice treated with or without hFIZZ3 indicate no acute change in glycemic control in response to hFIZZ3 administration.

In agreement with previously reported results for mResistin produced in the mammalian system (3, 35), we observed a significant decrease in lipid accumulation in human adipocytes treated with recombinant mResistin produced in *E. coli*. We found no effect of recombinant mResistin on proliferation or lipolysis in either human or mouse adipocytes, again consistent with the previously reported results (3, 35).

Our data raise the possibility that macrophage-secreted hFIZZ3 might have a different effect(s) on adipose functions from that of adipose-secreted mResistin. These differences between hFIZZ3 and mResistin could not be explained by the lack of species cross-reactivity, because both proteins have similar activities in human as well as mouse adipocytes. The divergence might have originated from the distinct surface receptors and/or intracellular signaling pathways affected by hFIZZ3 and mResistin. Preliminary data showing that, in contrast to mResistin (7, 9), hFIZZ3 does not inhibit insulin-stimulated Akt phosphorylation and activates ERK1/2 kinase suggest that hFIZZ3 may exert its activities via distinct signal transduction pathways from those of mResistin. The identification of their corresponding receptors will certainly help piece together the puzzle.

It is important to note that our recombinant hFIZZ3 affects adipocyte functions at concentrations that exceed the physiological circulating FIZZ3 level (11, 36, 37). It is possible that the recombinant hFIZZ3 used in our study has lower activity than that of endogenously produced hFIZZ3. Due to the lack of published data describing human FIZZ3 activity, we are unable to compare the functional potency of our recombinant protein with that of endogenous hFIZZ3. The crystal structure of mouse resistin shows that protein can form disulfide bond-dependent hexameric or trimeric complexes that might possess different activities (38). In serum, mResistin circulates in two distinct oligomeric forms that appear on SDS-PAGE as a dimer and a monomer, respectively. Although the tendency to form the disulfide-linked oligomers has been reported for recombinant human FIZZ3 produced in *E. coli* (39), and recombinant hFIZZ3 used in our study separates as a dimer and a monomer on SDS-PAGE, we cannot discount the possibility that bacterially expressed hFIZZ3 is unable to form correct multimeric assemblies, thereby reducing its activity. It is also possible that at the concentrations used in our study, hFIZZ3 activates a receptor(s) for another related ligand(s); thus, testing other human FIZZ proteins on human adipocytes may yield interesting findings. The fact that recombinant mResistin, similarly to hFIZZ3 produced in *E. coli*, is able to inhibit adipocyte differentiation similar to mResistin produced in the mammalian system suggests that *E.*

coli-produced hFIZZ3 and endogenous hFIZZ3 are likely to have similar activities (3, 35). Nevertheless, the functional identity between our recombinant and endogenous hFIZZ3 remains to be proven.

Based on high expression of hFIZZ3 in many fetal, but not adult, tissues, we speculate that hFIZZ3 play a regulatory role in progenitor cell proliferation during embryogenesis and may continue to support the need for tissue generation in response to certain physiological conditions in adults. The proliferative ability of hFIZZ3 demonstrated here supports this hypothesis. We propose that hFIZZ3 secreted from adipose-associated monocytes/macrophages might exert some beneficial effects on adipose tissue specifically during obesity. By increasing the number of preadipocytes, the progenitor cells for adipocytes that are ready to differentiate, hFIZZ3 might increase adipose lipid storage capacity. By increasing lipolysis, hFIZZ3 might provide a mechanism to convert lipid-overloaded hypertropic adipocytes to healthier, less lipid-containing cells. Finally, by accelerating the triglyceride/fatty acid futile cycle, hFIZZ3 might increase the metabolic rate and improve energy metabolism. Thus, hFIZZ3 may be a part of the mechanism that handles the increased need for functional adipose as well as for dissipating energy in the state of obesity.

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