Activation of Transient Receptor Potential Vanilloid Type-1 Channel Prevents Adipogenesis and Obesity

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Abstract—We tested the hypothesis that activation of transient receptor potential vanilloid type-1 (TRPV1) by capsaicin prevents adipogenesis. TRPV1 channels in 3T3-L1-preadipocytes and visceral adipose tissue from mice and humans were detected by immunoblotting and quantitative real-time RT-PCR. The effect of TRPV1 on cytosolic calcium was determined fluorometrically in 3T3-L1-preadipocytes and in human visceral fat tissue. Adipogenesis in stimulated 3T3-L1-preadipocytes was determined by oil red O-staining of intracellular lipid droplets, triglyceride levels, expression of peroxisome proliferator-activated receptor-γ, and expression of fatty acid synthase. Long-term feeding experiments were undertaken in wild-type mice and TRPV1 knockout mice.

We detected TRPV1 channels in 3T3-L1-preadipocytes and visceral adipose tissue from mice and humans. In vitro, the TRPV1 agonist capsaicin dose-dependently induced calcium influx and prevented the adipogenesis in stimulated 3T3-L1-preadipocytes. RNA interference knockdown of TRPV1 in 3T3-L1-preadipocytes attenuated capsaicin-induced calcium influx, and adipogenesis in stimulated 3T3-L1-preadipocytes was no longer prevented. During regular adipogenesis TRPV1 channels were downregulated which was accompanied by a significant and time-dependent reduction of calcium influx. Compared with lean counterparts in visceral adipose tissue from obese db/db and ob/ob mice, and from obese human male subjects we observed a reduced TRVP1 expression. The reduced TRPV1 expression in visceral adipose tissue from obese humans was accompanied by reduced capsaicin-induced calcium influx. The oral administration of capsaicin for 120 days prevented obesity in male wild type mice but not in TRPV1 knockout mice assigned to high fat diet. We conclude that the activation of TRPV1 channels by capsaicin prevented adipogenesis and obesity. **(*Circ Res*. 2007;100:1063-1070.)**

Key Words: transient receptor potential vanilloid type-1 ■ RNAi ■ TRPV1 knockout adipogenesis ■ obesity

Visceral obesity as clinically assessed by waist circumference depends on the proliferation and growth of preadipocytes which is closely regulated by several genes and extracellular factors.[1–3] Among these factors capsaicin ((E)-N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide) has been shown to affect lipid metabolism and obesity.[4,5] However, the underlying mechanisms by which capsaicin affect visceral adipose tissue have not been completely clarified yet. Recent studies indicated that capsaicin activates the transient receptor potential vanilloid type-1 (TRPV1) channel.[6–8] The capsaicin receptor TRPV1 belongs to the family of nonselective cation channels with high calcium permeability.[9]

Now, we tested the hypothesis that capsaicin-induced activation of TRPV1 in preadipocytes prevents adipogenesis and obesity. We showed that the activation of TRPV1 channels by capsaicin increased cytosolic calcium and prevented adipogenesis of preadipocytes in vitro. The effects of capsaicin on adipogenesis were attenuated after TRPV1 knockdown. Furthermore, capsaicin prevented TRPV1 downregulation during adipogenesis. Finally the administration of capsaicin prevented obesity in male wild-type mice but not in TRPV1 knockout mice assigned to high fat diet in vivo.

Materials and Methods

Detailed methods, sequences, and reagents used can be found in the online data supplement available at http://circres.ahajournals.org.

Culture of murine 3T3-L1 preadipocytes,[10–15] RNA interference technique,[16] measurements of cytosolic calcium,[17,18] immunoblottings, immunohistochemistry, determination of adipocyte size, PCR, and standardized techniques[17–19] were performed as described previously.

All mice (db/db mice, ob/ob mice, C57BL/6 wild-type mice, and TRPV1 knock-out mice) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Procedures were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

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Subjects were classified obese if waist circumference was >90 cm.[20,21] The protocol was approved by the local Ethics Committee. All patients gave written informed consent.

All values reported are mean±SEM. Comparisons between groups were analyzed using Student *t* test or one-way ANOVA with Bonferroni's multiple comparison post hoc test as appropriate (GraphPad Prism, La Jolla, Calif). Two-sided probability values <0.05 were considered to indicate statistical significance.

Results

First, we detected TRPV1 in 3T3-L1-preadipocytes and visceral adipose tissue from mice and humans by immunoblotting (Figure 1a) and immunohistochemistry (Figure 1b). The immunoblots confirmed the molecular mass of TRPV1 of 95 kDa and showed that the antibodies identified TRPV1 in both 3T3-L1-preadipocytes and visceral adipose tissue. Immunohistochemistry demonstrated specific staining for TRPV1 in the cell membrane of 3T3-L1-preadipocytes and adipocytes from mice and humans.

Caterina et al[6] and Savidge et al[22] showed that the specific agonist capsaicin induces calcium influx through TRPV1 channels. To confirm the functional properties of TRPV1 channels in 3T3-L1-preadipocytes intracellular calcium concentrations were measured using the fluorescent dye technique. We used 3T3-L1-preadipocytes because they are an established model for characterizing the events responsible for adipocyte differentiation.[10,12] As shown in Figure 1c, the TRPV1 agonist capsaicin dose-dependently increased intracellular calcium in fura-2 loaded 3T3-L1-preadipocytes. The capsaicin-induced calcium increase could be observed at a concentration of 10 nmol/L, whereas the maximum effect was obtained at 1 μmol/L.

Based on previous findings by Miller et al[10] and Shi et al[11] that a calcium increase inhibits differentiation of preadipocytes we now evaluated the effect of capsaicin on adipogenesis of 3T3-L1-preadipocytes. Adipogenesis was induced in 3T3-L1-preadipocytes using 3-isobutyl-1-methylxanthine, dexamethasone, and insulin as recommended.[12,13] The differentiation of preadipocytes was examined by four different functional tests, including oil red O-staining of intracellular lipid droplets,[13] determination of triglyceride levels, expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), a key transcriptional regulator of adipogenesis,[14] and the expression of fatty acid synthase, a key enzyme in de novo lipogenesis.[15] Increased oil red O-staining of lipid droplets in cells indicated a time-dependent adipocyte differentiation after induction of adipogenesis with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (Figure 1d). After induction of adipogenesis cells differentiated into morphologically distinct, fat-laden adipocytes with accumulated cytoplasmic triglycerides that stained with oil red O. Under control conditions increased lipid droplets could be observed approximately 3 days after induction of adipogenesis. In the presence of the TRPV1 agonist capsaicin (final concentration, 1μmol/L) we observed a reduced oil red O-staining of cells after the induction of adipogenesis. In contrast, administration of the TRPV1 antagonist capsazepine did not prevent adipogenesis in 3T3-L1-preadipocytes.

Determination of cellular triglyceride levels confirmed that capsaicin prevented adipogenesis. Compared with control conditions the induction of adipogenesis for 8 days significantly increased triglyceride levels from 3.4±0.2 mmol/L to 10.8±0.8 mmol/L in 3T3-L1-preadipocytes (each n=5; $P<0.01$). The triglycerides levels after induction of adipogenesis for 8 days in the presence of capsaicin were significantly lower compared with the values in the absence of capsaicin (6.2±0.4 mmol/L versus 10.8±0.8 mmol/L; each n=5; $P<0.01$). In contrast, administration of the TRPV1 antagonist capsazepine did not prevent the increase of triglyceride levels in 3T3-L1-preadipocytes during adipogenesis (12.3±0.6 mmol/L; n=5). Compared with control conditions the induction of adipogenesis for 8 days significantly increased the expression of PPAR-γ from 1.00±0.06 to 2.02±0.01 in 3T3-L1-preadipocytes (each n=5; $P<0.01$). The expression of PPAR-γ after induction of adipogenesis for 8 days in the presence of capsaicin was significantly lower compared with the values in the absence of capsaicin (1.18±0.16 versus 2.02±0.01; each n=5; $P<0.01$). In contrast, administration of the TRPV1 antagonist capsazepine did not prevent the increased expression of PPAR-γ in 3T3-L1-preadipocytes during adipogenesis (1.75±0.12; n=5). Finally, compared with control conditions the induction of adipogenesis for 8 days significantly increased the expression of fatty acid synthase from 1.01±0.08 to 2.99±0.09 in 3T3-L1-preadipocytes (each n=4; $P<0.01$). The expression of fatty acid synthase after induction of adipogenesis for 8 days in the presence of capsaicin was significantly lower compared with the values in the absence of capsaicin (1.17±0.10 versus 2.99±0.09; each n=4; $P<0.01$). In contrast, administration of the TRPV1 antagonist capsazepine did not prevent the increased expression of fatty acid synthase in 3T3-L1-preadipocytes during adipogenesis (2.86±0.15; n=4).

As negative controls we showed that without the induction of adipogenesis by 3-isobutyl-1-methylxanthine, dexamethasone, and insulin neither capsaicin nor capsazepine had a significant effect on the production of lipid droplets in 3T3-L1-preadipocytes (Figure 1d). Without induction of adipogenesis triglyceride levels in 3T3-L1-preadipocytes were similar at day 8 under control conditions (3.4±0.2mmol/L), in the presence of capsaicin (3.3±0.2mmol/L) or capsazepine (3.7±0.3 mmol/L; each n=5; p=n.s.). Without induction of adipogenesis expression of PPAR-γ in 3T3-L1-preadipocytes was similar at day 8 under control conditions (1.00±0.06), in the presence of capsaicin (1.23±0.03) or capsazepine (1.00±0.04; each n=5; p=n.s.). Finally, without induction of adipogenesis expression of fatty acid synthase in 3T3-L1-preadipocytes was similar at day 8 under control conditions (1.01±0.08), in the presence or capsaicin (1.10±0.09) or capsazepine (0.99±0.10; each n=4; p=n.s.). From these experiments it is concluded that the capsaicin-induced calcium increase through TRPV1 channels prevented adipogenesis.

During adipogenesis of 3T3-L1-preadipocytes induced by 3-isobutyl-1-methylxanthine, dexamethasone, and insulin a time-dependent downregulation of TRPV1 expression could be observed by immunoblotting (Figure 1e). The downregulation of TRPV1 expression during adipogenesis was prevented in the presence of the TRPV1 agonist capsaicin. Eight days after induction of adipogenesis in 3T3-L1-preadipocytes, TRPV1 channel expression was significantly reduced from control value of 1.00±0.08 to 0.46±0.03 (each n=4; $P<0.01$), whereas it was 1.35±0.03 (n=4; $P<0.01$ compared with control) when the

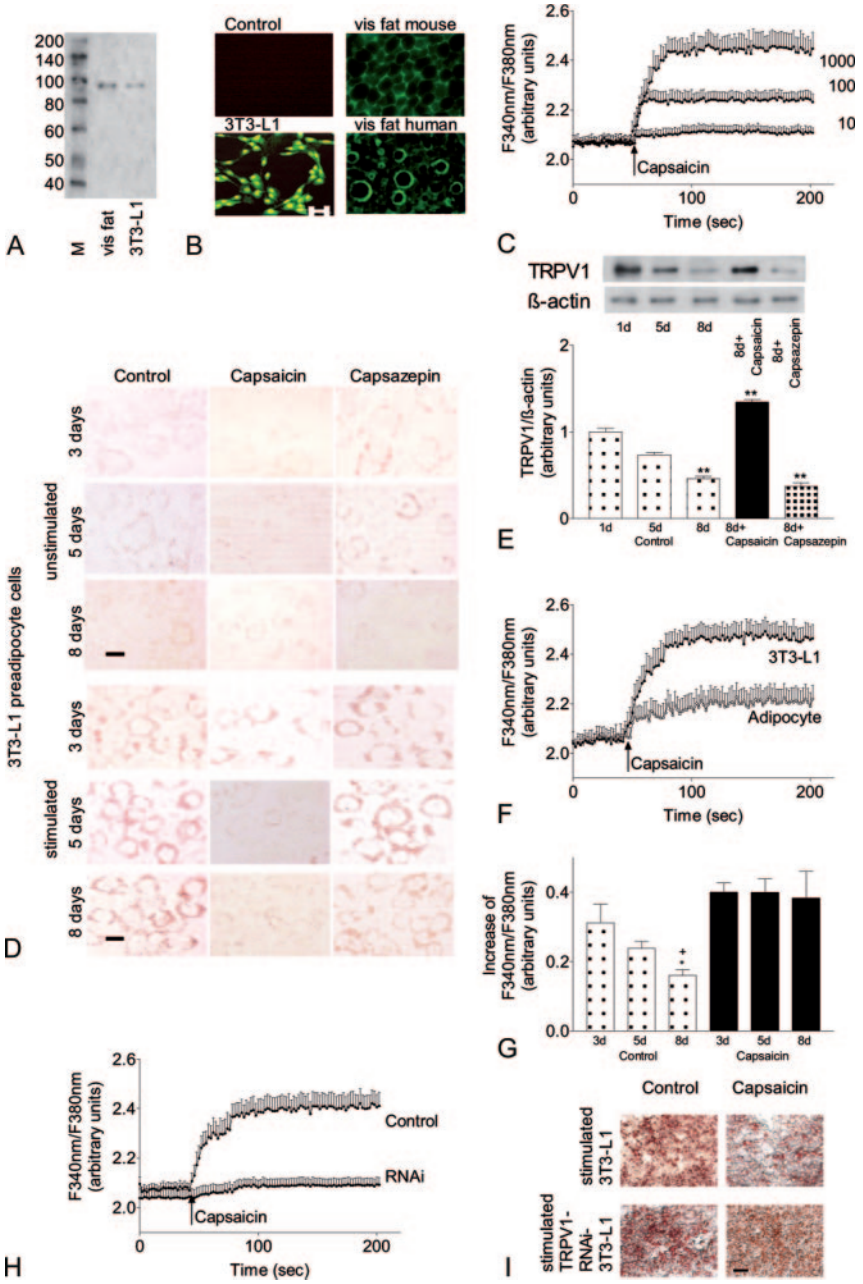

Figure 1. The TRPV1 channel agonist capsaicin increases calcium, prevents adipogenesis, and prevents downregulation of TRPV1 channels in 3T3–L1-preadipocytes. a, Immunoblot of TRPV1 obtained from 3T3–L1-preadipocytes (3T3–L1), from visceral adipose tissue (vis fat). The predicted molecular weight of TRPV1 is 95 kDa. M denotes marker. Immunoblots are representative of 3 separate experiments showing similar results. b, Immunohistochemical staining of 3T3–L1-preadipocytes (3T3–L1), visceral adipose tissue (vis fat) from mouse and from humans with anti-TRPV1 antibody showing TRPV1 on the membrane surface. A negative control (only second antibody added) is shown in upper left panel. For 3T3–L1-preadipocytes an overlay with the nucleus stained using PI (red color) and TRPV1 stained in green is shown. Panels are representative of 3 separate experiments showing similar results. Bar denotes 10 μm. c, Concentration-dependent increase of cytosolic free calcium concentration in 3T3–L1-preadipocytes after addition of capsaicin. Fura2-loaded 3T3–L1-preadipocytes were stimulated with increasing capsaicin concentrations (in nmol/L) as indicated beside each tracing and the fluorescence was measured at 510 nm emission with excitation wavelengths of 340 nm and 380 nm. Data are mean±SEM from 6 separate experiments. d, Capsaicin prevents adipogenesis in 3T3–L1-preadipocytes. Identification of adipogenesis in 3T3–L1-preadipocytes in the absence (control) and presence of TRPV1 agonist capsaicin or TRPV1 antagonist capsazepine. Lipid droplets were visualized by oil red O-staining. Upper panels were obtained without induction of adipogenesis of 3T3–L1-preadipocytes (unstimulated), whereas lower panels were obtained after induction of adipogenesis of 3T3–L1-preadipocytes for several days (stimulated) as indicated. Scale bar indicates 10 μm; magnification, ×400. Representative pictures of 3 separate experiments are shown. e, Capsaicin prevents the downregulation of TRPV1 expression during adipogenesis of 3T3–L1-preadipocytes. Adipogenesis was induced in the absence (control) and presence of TRPV1 agonist capsaicin or TRPV1 antagonist capsazepine for 8 days. TRPV1 expression in 3T3–L1-preadipocytes was measured at the indicated days. Data are mean±SEM. $^{**}P<0.01$ compared with control (absence of capsaicin, day 1). f, Reduced capsaicin-induced calcium influx into adipocytes compared with 3T3–L1-preadipocytes. 3T3–L1-preadipocytes were cultured without induction of adipogenesis (unstimulated) and with induction of adipogenesis (stimulated) to generate adipocytes for 8 days. Fluorescence tracings after addition of 1 μmol/L capsaicin to fura2-loaded 3T3–L1-preadipocytes or fura2-loaded adipocytes are shown. Data are mean±SEM from 6 separate experiments. g, Time-dependent reduction of capsaicin (1 μmol/L)-induced calcium influx during adipogenesis. Calcium influx into 3T3-L1-preadipocytes which were cultured with induction of adipogenesis in the absence (control) and presence of capsaicin was measured at different time points as indicated. Data are mean±SEM from 6 separate experiments. $^{*}P<0.05$ compared with control (absence of capsaicin, day 3). $+P<0.05$ compared with capsaicin (day 8). h, RNAi knockdown of TRPV1 reduces capsaicin-induced calcium influx into 3T3-L1-preadipocytes. Fluorescence tracings after addition of 1μmol/L capsaicin to fura2-loaded 3T3-L1-preadipocytes after control transfection (Control) and after transfection with RNAi against TRPV1 (RNAi). Data are mean±SEM from 6 separate experiments. i, Capsaicin did not prevent adipogenesis in TRPV1 knockdown 3T3-L1-preadipocytes. 3T3-L1-preadipocytes after control transfection (3T3-L1) and after transfection with RNAi against TRPV1 (TRPV1-RNAi-3T3-L1) were stimulated with by 3-isobutyl-1-methylxanthine, dexamethasone, and insulin in the absence (control) and presence of TRPV1 agonist capsaicin. Scale bar indicates 10 μm; magnification, ×200. Representative pictures of 3 separate experiments are shown.

induction of adipogenesis was performed in the presence of capsaicin. The administration of the TRPV1 antagonist capsazepine did not prevent the downregulation of TRPV1 channel after induction of adipogenesis in 3T3-L1-preadipocytes.

The capsaicin-induced calcium increase was significantly lower in mature adipocytes compared with 3T3-L1-preadipocytes (32±4%, versus 100±11%; each n=10; $P<0.01$; Figure 1f). In accordance with the observed downregulation of TRPV1 chan-

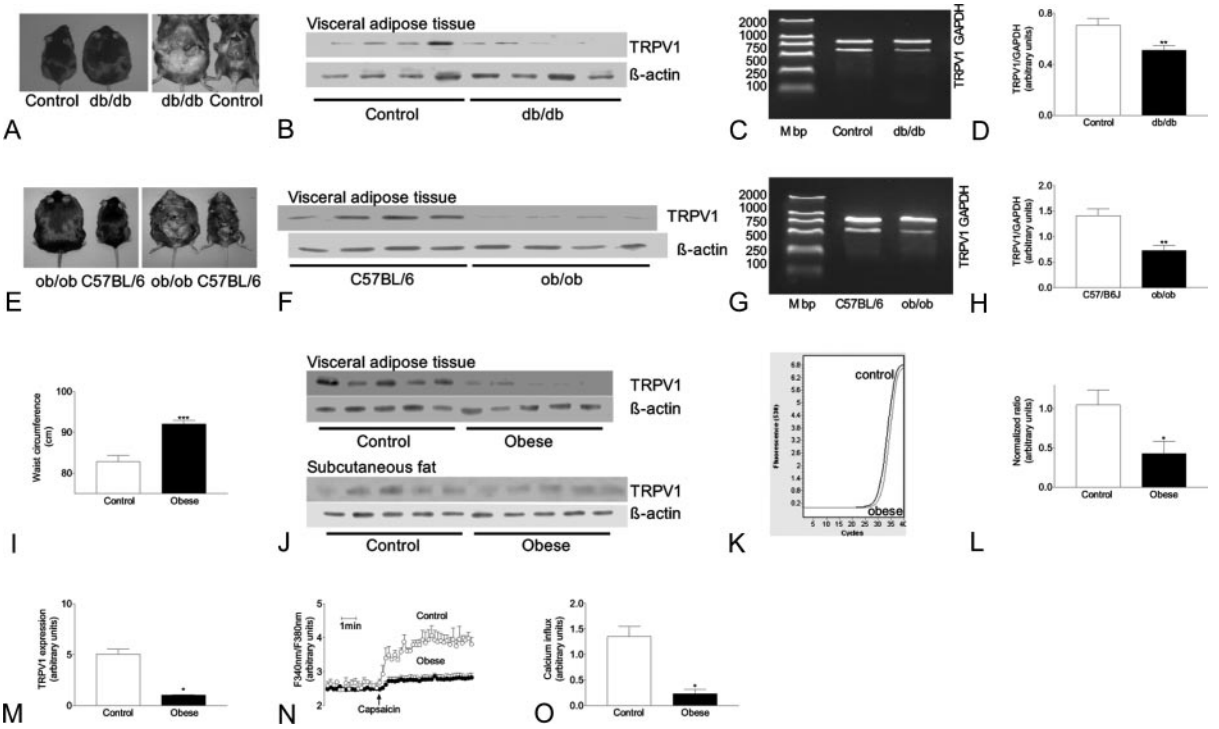

Figure 2. Reduced TRPV1 expression in visceral adipose tissue from obese db/db mice (upper panel), obese ob/ob mice (middle panel) and obese human male subjects (lower panel). Picture of body shape and abdominal situs showing visceral fat (a and e); expression of TRPV1 in visceral adipose tissue (b and f); representative agarose gel electrophoresis of PCR products from mRNA of TRPV1 and GAPDH genes in visceral adipose tissue (c and g), M denotes bp marker; and summary data (d and h); each n=4. $^{**}P<0.01$ between groups. Lower panels show data from age-matched human male control subjects and obese human male subjects. Waist circumference (i); each n=8. $^{***}P<0.001$ between groups; expression of TRPV1 in visceral adipose tissue and subcutaneous fat (j); representative quantitative real-time RT-PCR from visceral adipose tissue (k) from 1 human male control subject (black line) and from 1 obese human male subject (red line); y-axis denotes fluorescence at 530 nm in arbitrary units, and x-axis denotes number of cycles of the quantitative real-time RT-PCR; The shift of the red line to the right compared with the black line indicates reduced TRPV1 transcripts in obese subjects; summary data of quantitative real-time RT-PCR are given in (l); n=4. $^{*}P<0.05$ between groups. Quantitative in-cell Western assay of TRPV1 expression in visceral adipose tissue from 1 human control subject (open circles) and 1 obese human subject (filled circles) (n); and summary data (o); each n=4; $^{*}P<0.05$).

nel expression during adipogenesis, we observed a significant and time-dependent reduction of calcium influx during adipogenesis. On the other hand, the calcium influx was unchanged when adipogenesis was blocked in the presence of capsaicin (Figure 1g). Resting cytosolic calcium concentration was not significantly different in mature adipocytes compared with 3T3-L1-preadipocytes or stimulated 3T3-L1-preadipocytes in the presence of capsaicin (104±2 nmol/L; 101±4 nmol/L; 105±3 nmol/L; each n=12; p=n.s.), probably indicating long-lasting increased calcium extrusion after transient rise in intracellular calcium.

To verify that TRPV1 channels are involved in capsaicin-induced calcium increase in 3T3-L1-preadipocytes and capsaicin-dependent prevention of adipogenesis, TRPV1 knockdown was performed by gene silencing with RNA interference using specific RNA interference (RNAi).[16] Compared with empty-control-transfected 3T3-L1-preadipocytes the TRPV1 expression in RNAi-transfected 3T3-L1-preadipocytes was significantly reduced to 38±3% (n=3; $P<0.05$). The thereby reduced TRPV1 channel expression caused a significant reduction of capsaicin-induced calcium increase from 100±16% in empty-control-transfected 3T3-L1-preadipocytes to 16±3% in TRPV1 RNAi-transfected 3T3-L1-preadipocytes (n=10; $P<0.01$; Figure 1h). Adipogenesis was induced in TRPV1 knockdown 3T3-L1-preadipocytes and control-transfected 3T3-L1-preadipocytes by 3-isobutyl-1-methylxanthine, dexamethasone, and insulin and adipogenesis was evaluated by oil red O-staining of lipid droplets (Figure 1i). As expected, in TRPV1 knockdown 3T3-L1-preadipocytes the administration of capsaicin did not prevent adipogenesis, whereas in control-transfected 3T3-L1-preadipocytes the TRPV1 agonist capsaicin prevented adipogenesis. These experiments confirmed the necessity of TRPV1 channel activation in capsaicin-dependent prevention of adipogenesis.

Now the question arises, whether downregulation of TRPV1 channels is a common finding in several animal models of obesity as well as in human obesity. We therefore investigated TRPV1 channel expression using immunoblotting and mRNA detection in visceral adipose tissue from genetically obese db/db mice, which have a nonfunctional leptin receptor, and from obese ob/ob mice, which have a naturally occurring leptin deletion.[23] As shown in Figure 2a through 2d, genetically obese db/db mice (body weight, 27±1g; abdominal circumference, 8.1±0.1 cm; visceral fat, 1.6±0.1 g) had significantly lower TRPV1 channel expression (Figure 2b) and TRPV1 mRNA (Figure 2c; 0.50±0.01 versus 0.71±0.05; each n=6; $P<0.01$) in

visceral adipose tissue compared with their lean littermates (body weight, 16±1g; abdominal circumference, 6.1±0.3 cm; visceral fat, 0.3±0.1 g). As shown in Figure 2e through 2h, leptin-deficient obese ob/ob mice (body weight, 50±4 g; abdominal circumference, 11.6±0.1 cm; visceral fat, 5.0±0.4 g) had significantly lower TRPV1 channel expression (Figure 2f) and TRPV1 mRNA (Figure 2g; 0.73±0.10 versus 1.41±0.13; each n=6; $P<0.01$) in visceral adipose tissue compared with lean C57BL/6 counterparts (body weight, 25±1 g; abdominal circumference, 7.9±0.2 cm; visceral fat, 0.6±0.2 g).

We further investigated TRPV1 channel expression in adipose tissue from human male subjects. Visceral adipose tissue and subcutaneous fat was obtained during cholecystectomy. Subjects were classified obese if waist circumference was more than 90 cm according to the Asian WHO criteria.[20,21] Eight lean male control subjects (age, 43±1 years; body mass index, 24.4±1.4 kg/m^2, serum triglycerides, 4.6±0.4 mmol/L) had a waist circumference of 82.8±1.5 cm, whereas 8 age-matched obese male subjects (age, 44±2 years, body mass index, 26.3±0.4 kg/m^2, serum triglycerides, 4.9±0.6 mmol/L) had a significantly higher waist circumference of 92.0±0.8 cm ($P<0.001$ for the waist circumference between the groups; Figure 2i). Western blot shows that obese human male subjects had significantly lower TRPV1 channel expression in visceral adipose tissue (0.70±0.15 versus 4.93±1.07; each n=8; $P<0.01$) and subcutaneous fat (1.22±0.08 versus 1.70±0.13; each n=8; $P<0.01$) compared with age-matched lean human male control subjects (Figure 2j). Using quantitative real-time RT-PCR from visceral adipose tissue (Figure 2k and 2l) we showed that obese human male subjects had significantly reduced TRPV1 mRNA compared with age-matched lean human male control subjects (normalized ratio, 0.43±0.16 versus 1.05±0.19, n=4; $P<0.05$).

Next we evaluated whether reduced TRPV1 expression is also linked to reduced calcium influx in visceral adipose tissue from 4 control subjects (body mass index, 23.5±0.3 kg/m^2) and 4 age-matched obese subjects (body mass index, 32.4±2.0 kg/m^2). Normalized TRPV1 protein expression by quantitative in-cell Western assay in visceral adipose tissue was significantly lower in obese subjects compared with control subjects (normalized TRPV1 expression, 1.02±007 versus 5.05±0.49; each n=4, $P<0.05$; Figure 2m). The capsaicin-induced calcium influx was significantly lower in obese subjects compared with control subjects (0.2±0.1 versus 1.3±0.2; each n=4; $P<0.05$; Figure 2n and 2o).

These findings indicated that downregulation of TRPV1 channel in visceral adipose tissue is a common finding for obesity. Hence a putative intervention which activates TRPV1 channels to prevent obesity should start early during adipogenesis.

As proof of principle we now evaluated whether continuous activation of TRPV1 channels by capsaicin can prevent obesity, hypertrophy of adipocytes, and prevent downregulation of TRPV1 channels in male mice assigned to high fat diet. We first analyzed the biometrical characteristics of mice on control diet and high fat diet, both in the absence and presence of TRPV1 channel agonist capsaicin (Figure 3). After 120 days mice on high fat diet had significantly higher body weight compared with control mice (44±1 g versus 29±1 g; each n=10; $P<0.01$). Mice on high fat diet plus capsaicin remained lean (31±1 g; n=10; $P<0.01$ compared with mice on high fat diet). Body weight of mice on control diet plus capsaicin was not significantly different to mice on control diet (27±1 g versus 29±1 g; each n=10; p=n.s.). As shown in Figure 3c we observed a decrease in food intake within the first days after starting capsaicin administration. However, 10 days after starting capsaicin administration the food intake was not significantly different between the groups (food intake per mouse per day in control mice, 4.6±0.1 g; mice on high fat diet, 4.8±0.1 g; control mice with capsaicin, 4.8±0.1; mice on high fat diet with capsaicin, 4.7±0.1 g; p=n.s. by ANOVA). These data indicated that long term feeding with capsaicin did not change food intake. Mice on high fat diet showed obesity with significantly increased waist circumference and increased plasma triglyceride levels compared with control mice or mice on high fat diet plus capsaicin. Mice on high fat diet had significantly more visceral adipose tissue, subcutaneous fat and brown fat compared with control mice or mice on high fat diet plus capsaicin (Figure 3d through 3i). These data indicated that the administration of the TRPV1 agonist capsaicin prevented obesity in mice on high fat diet.

Figure 4 shows adipocyte size and TRPV1 channel expression in adipose tissue in mice. Mice on high fat diet had significantly increased adipocyte size in visceral adipose tissue compared with control mice (3708±199 μm^2, n=78; versus 1062±37 μm^2, n=101 $P<0.001$). Mice on high fat diet plus capsaicin had small adipocytes size (1255±64 μm^2, n=90; $P<0.001$ compared with mice on high fat diet). Similar findings could be obtained in subcutaneous fat and brown fat (Figure 4b through 4d). Mice on high fat diet had significantly reduced TRPV1 channel expression in visceral adipose tissue compared with control mice (0.44±0.06 versus 1.00±0.05; each n=10; $P<0.001$). Furthermore, mice on high fat diet plus capsaicin had significantly increased TRPV1 channel expression in visceral adipose tissue (1.18±0.08, n=10; $P<0.001$) compared with mice on high fat diet (Figure 4e). The administration of capsaicin increased TRPV1 channel expression in visceral adipose tissue by 169%. As shown in Figure 4f mice on high fat diet had reduced TRPV1 mRNA in visceral adipose tissue compared with control mice. Furthermore, mice on high fat diet plus capsaicin had increased TRPV1 mRNA in visceral adipose tissue compared with mice on high fat diet, supporting the protein expression data.

As appropriate controls TRPV1 knockout mice were exposed to high fat diet in the presence and absence of capsaicin. As could be predicted from the findings presented above, in TRPV1 knockout mice on high fat diet for 60 days the body weight was not significantly different in the absence and presence of the TRPV1 agonist capsaicin (30.3±0.7 g versus 29.8±0.4 g; each n=3; p=n.s.). In contrast wild-type mice on high fat diet for 60 days had a body weight of 32.0±0.4 g, whereas wild-type mice on high fat diet plus capsaicin a significantly reduced body weight of 27.2±1.3 g (each n=5; $P<0.05$ compared with mice on high fat diet). These data strongly support the hypothesis that the preventive effects of capsaicin are mediated by the TRPV1 channel.

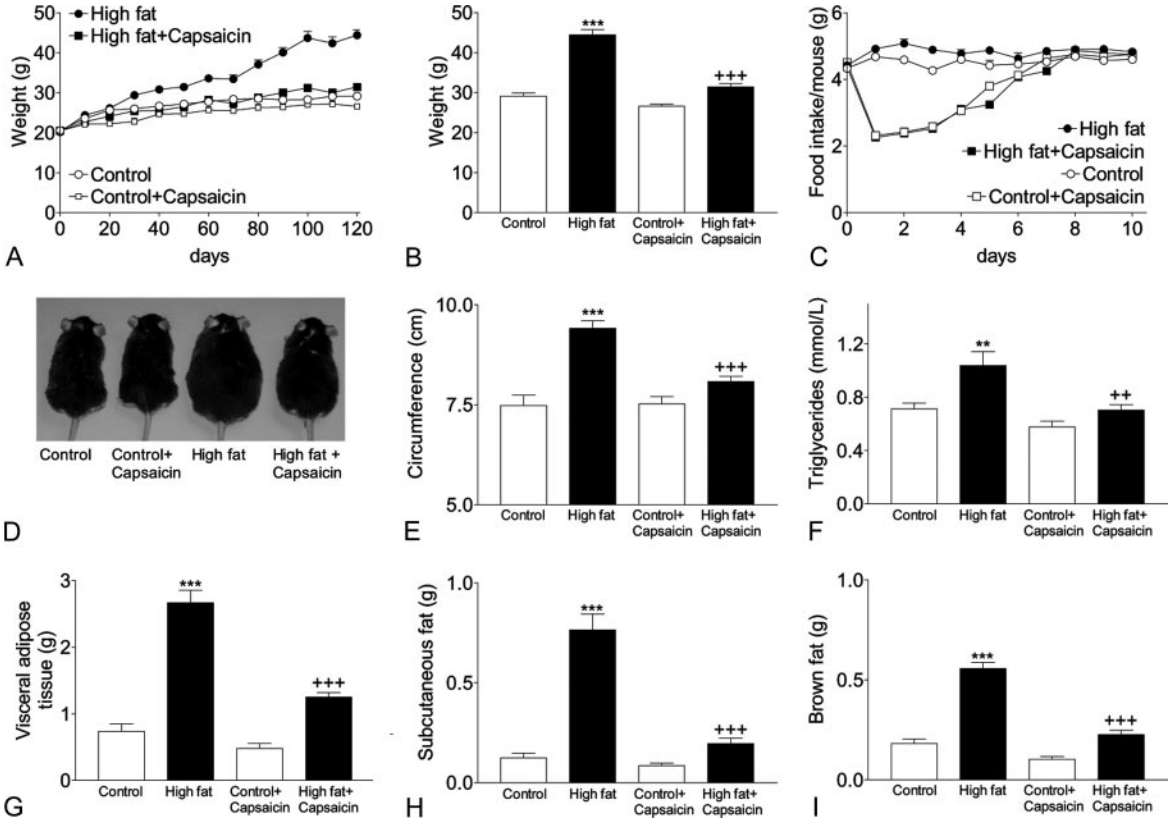

Figure 3. The TRPV1 channel agonist capsaicin prevents obesity in mice on high fat diet. a, Time course of body weight from control mice (open circles), mice on high fat diet (filled circles), control mice with capsaicin (open squares), and mice on high fat diet with capsaicin (filled squares; each n=10); Body weight (b), daily food intake per mouse during the first 10 days after the start of capsaicin administration in control mice (open circles), mice on high fat diet (filled circles), control mice with capsaicin (open squares), and mice on high fat diet with capsaicin (filled squares; each n=10) (c), picture of body shape of mice (d), waist circumference (e), serum triglycerides (f), visceral adipose tissue (g), subcutaneous fat (h), brown fat (i) at the age of 120 days of control mice (n=10), mice on high fat diet (n=10), control mice plus capsaicin (n=10), and mice on high fat diet plus capsaicin (n=10). $^{**}P<0.01$ for the comparison high fat vs control; $++P<0.01$ for the comparison high fat plus capsaicin vs high fat.

Discussion

TRPV1 channels have been described in several tissues.[24] This study for the first time detected TRPV1 transcripts and channel protein in 3T3-L1-preadipocytes and adipose tissue both in mice and humans. A limitation of the present study is that although we observed reduced TRPV1 expression both on transcript level and on protein level in two obese animal models and in obese humans compared with lean counterparts it is yet unknown whether there is a linear association between body mass index or waist circumference and TRPV1 expression levels. This would need larger cohort studies which may be difficult because of the limited availability of human visceral adipose tissue.

The TRPV1 agonist capsaicin dose-dependently increased intracellular calcium in 3T3-L1-preadipocytes. After TRPV1 knockdown by gene silencing with RNA interference using specific RNAi the capsaicin-induced calcium increase was significantly attenuated in 3T3-L1-preadipocytes. The capsaicin-induced calcium influx through TRPV1 channels has already been reported in HEK293 cells transfected with TRPV1 channels, neurons, and hepatoblastoma cells.[6,22,25] Furthermore, capsaicin had several effects on nonneuronal tissues.[26] Capsaicin activates migration of human polymorphonuclear cells and elicited a substantial constriction in isolated arterioles which may be related to capsaicin-induced transplasmamembrane calcium influx.[26,27] As detailed in a review by Szallasi and Blumberg, capsaicin is a known specific agonist of TRPV1 channels.[26] The potency of capsaicin-induced calcium influx was 320 nmol/L in neurons, 660 nmol/L in mast cells and 380 nmol/L in glioma cells.[26] In our study the capsaicin-induced calcium influx was dose-dependent and thus confirmed the functional properties of TRPV1 channels in preadipocytes. It should be noted that capsaicin concentrations as low as 10nmol/L showed calcium influx in preadipocytes. The characteristics of capsaicin-induced calcium entry through TRPV1 channels was similar to that reported by Caterina et al[6] and Savidge et al[22] and matches the characteristics of the TRPV1 channel.

Previous studies indicated that adipogenesis is regulated by calcium.[10,11] Shi et al reported that elevated calcium markedly suppressed adipogenesis with a decrease in triglyceride accumulation and a substantial inhibition in PPAR-γ expression.[11] In the present study adipogenesis was induced in 3T3-L1-preadipocytes using 3-isobutyl-1-methylxanthine, dexamethasone, and insulin.[12,13] We showed that capsaicin-induced elevation of cytosolic calcium reduced intracellular lipid droplets, reduced triglyceride levels, reduced expression of PPAR-γ and reduced fatty acid synthase in stimulated

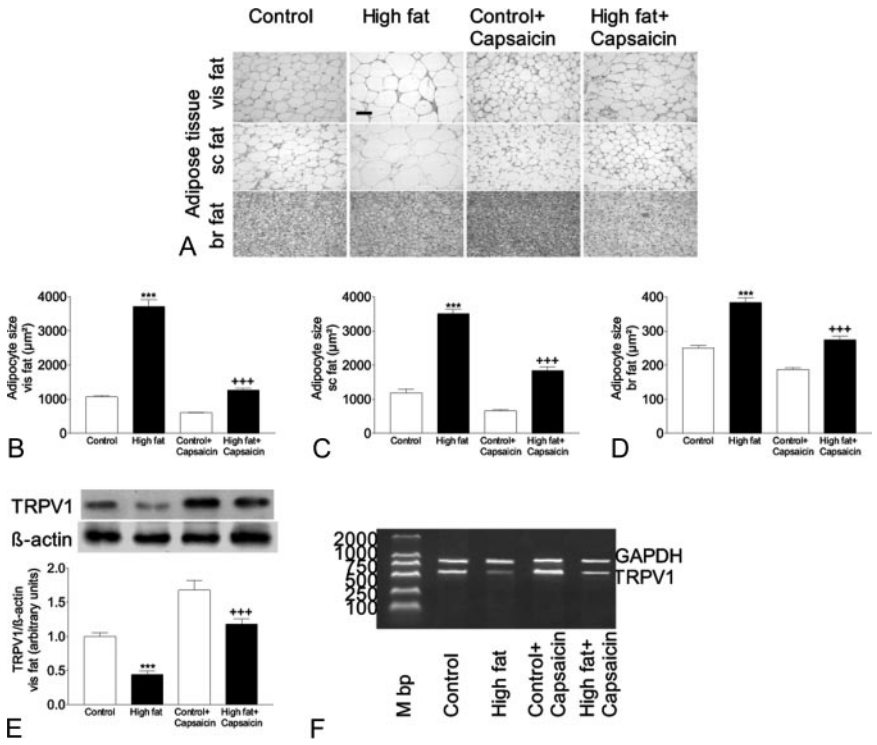

Figure 4. The TRPV1 channel agonist capsaicin prevents adipocyte hypertrophy and the reduction of TRPV1 expression in visceral adipose tissue from mice on high fat diet. a, Representative cross sections of visceral adipose tissue (vis fat), subcutaneous fat (sc fat), and brown fat (br fat) from control mice, mice on high fat diet, control mice plus capsaicin, and mice on high fat diet plus capsaicin. Scale bar indicates 10 μm; magnification, $\times$400. Summary data of adipocyte size of visceral adipose tissue (b), subcutaneous fat (c), and brown fat (d) in mice. $^{***}P<0.001$ for the comparison high fat vs control; $+++P<0.001$ for the comparison high fat plus capsaicin vs high fat. e, Expression of TRPV1 in visceral adipose tissue (vis fat) from mice. $^{***}P<0.001$ for the comparison high fat vs control; $+++P<0.001$ for the comparison high fat plus capsaicin vs high fat. f, Representative agarose gel electrophoresis of PCR products from mRNA of TRPV1 and GAPDH genes in visceral adipose tissue mice M denotes bp marker. Panels are representative of 3 separate experiments showing similar results.

preadipocytes, whereas administration of the TRPV1 antagonist capsazepine did not prevent adipogenesis. Furthermore, after TRPV1 knockdown the administration of capsaicin did not prevent adipogenesis in TRPV1 knockdown 3T3-L1-preadipocytes. Hence we confirmed that increased calcium after administration of capsaicin significantly blocked adipogenesis in stimulated 3T3-L1-preadipocytes.[11,28,29]

As proof of principle the effect of capsaicin on adipogenesis and obesity was then investigated in vivo, both in control mice and in TRPV1 knockout mice. Our long term feeding experiments showed that the administration of capsaicin prevented obesity in male wild-type mice assigned to high fat diet but not in TRPV1 knockout mice assigned to high fat diet, indicating that TRPV1 is directly involved in adipogenesis and obesity in vivo.

Capsaicin has been reported to reduce adiposity in rats and mice, which was partly explained by its effect on energy and lipid metabolism via catecholamine secretion from the adrenal medulla through sympathetic activation of the central nervous system. However, these effects on catecholamine secretion were observed after intravenous administration of capsaicin in anesthetized rats.[30] It is unclear whether these mechanisms may also explain the long term effects of oral administration of capsaicin on obesity. Furthermore, sympathetic activity has shown consistent elevation in obesity, whereas catecholamine levels in obesity have been conflicting, with high, normal, and low levels reported.[31]

In the present long term feeding study changes of food intake in the presence of capsaicin have been ruled out by measurements of food intake. We showed a decrease in food intake within the first few days after starting the administration of capsaicin confirming a recent study by Wang et al who reported reduced short term food intake after TRPV1 activation of sensory neurons.[32] We showed that 10 days after starting capsaicin administration the food intake was similar in the presence and absence of capsaicin, which is in accordance with findings in obese Zucker rats.[33] An effect of red pepper on thermogenesis has been described.[4] However, several evidences indicated that afferent reflexes are mainly responsible for diet-induced thermogenesis.[34–36]

Using normal diet, Rong et al did not report a significant difference of feeding behavior, growth rate and body weight between TRPV1 knockout mice and wild-type mice.[37] However, no long term feeding studies comparing high fat diet with normal diet have yet been reported in TRPV1 knockout mice. In the present study we showed for the first time that capsaicin did not prevent adipogenesis and obesity in TRPV1 knockout mice on high fat diet.

In conclusion we present in vitro and in vivo evidences that capsaicin-induced calcium influx through TRPV1 channels prevents adipogenesis, prevents downregulation of TRPV1 expression and finally prevents obesity.

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Disclosures

None.

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Activation of Transient Receptor Potential Vanilloid Type-1 Channel Prevents Adipogenesis and Obesity

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Activation of transient receptor potential vanilloid type-1 (TRPV1) channel prevents adipogenesis and obesity

Online Data supplement

Materials and Methods

Cell culture and adipocyte differentiation assay

Murine 3T3-L1 preadipocytes as previously described[1-4] were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone) containing 100 μg/ml penicillin and 100 μg/ml streptomycin (GIBCO). Cells were plated and grown until 2 days post-confluence. Differentiation was then induced (day 0) by changing the medium to Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 μmol/L dexamethasone, and 5 μg/mL insulin.[3,4] Differentiation was performed in the absence and presence of 1 μmol/L capsaicin or 1 μmol/L capsazepine as indicated. Capsaicin or capsazepine were continuously present during the cell culture if no indicated otherwise. Cells were cultured, fixed and stained with the lipophilic dye oil red O (Sigma-Aldrich). Red staining shows lipid droplets in the cytoplasm indicating adipocyte differentiation.[4] Stained cells were either photographed or counterstained with Giemsa and visualized by bright field microscopy. The viability of cells after administration of capsaicin was checked using the trypan blue exclusion method and showed a cell viability more than 95%. The differentiation of preadipocytes was further examined by determination of triglyceride levels in cells, and by immunoblottings of the expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ), a key transcriptional regulator of adipogenesis,[5] and the expression of fatty acid synthase, a key enzyme in de novo lipogenesis.[6]

Selective silencing of TRPV1 by RNA interference

Selective silencing of TRPV1 by RNA interference using a lentiviral system was performed according the reported technique.[7] The lentiviral system includes three packaging plasmids (pLP1, pLP2, and pLP-VSVG) and one expression plasmid (PLL3.7). In order to select the cells infected with lentivirus, we replaced the reporter gene (green fluorescent protein, EGFP) of PLL3.7 by the Neo gene (called, PLL3.7-Neo). Small hairpin oligonucleotides DNA were synthesized and targeted the following sequences in TRPV1 cDNA, (1)GACAGATAGCCTGAAGCAG; and (2) GCGCATCTTCTACTTCAAC. Two oligonucleotides DNA were synthesized:

M547-569:

P1:5'-TGACAGATAGCCTGAAGCAGTTCAAGAGACTGCTTCAGGCTATCTGTCTTTTTTC-3';

P2:5'-TCGAGAAAAAAGACAGATAGCCTGAAGCAGTCTCTTGAACTGCTTCAGGCTATCTGTCA-3'

M1294-1316:

H1:5'-TGCGCATCTTCTACTTCAACTTCAAGAGAGTTGAAGTAGAAGATGCGCTTTTTTC-3'

H2:5'-TCGAGAAAAAAGCGCATCTTCTACTTCAACTCTCTTGAAGTTGAAGTAGAAGATGCGCA-3'

HpaI-XhoI were used to linearize the PLL3.7 expression plasmid. Recombined expression plasmids PLL3.7-Neo-(1) and PLL3.7-Neo-(2) were constructed by linking the small hairpin DNA-(1) and DNA-(2) with the linear vector. The PLL3.7 plasmid was used as negative control. The recombinant plasmids were identified by digestion with XhoI-XbaI and DNA sequence analysis.

Viral production was performed in 293FT cell. 1.5 μg packaging plasmids, 0.5 μg recombinant expression plasmids and 6 μL lipofectamine 2000 were added into 500 μL Opti-MEM (antibiotics free), mixed gently and incubated for 20 minutes at room temperature. Then the DNA-lipofectamine complexes and 1 mL of the 293FT cell suspension (a total of 10^6 cells) were added to the plate containing 1 mL of Opti-MEM (antibiotics free), and incubated overnight at 37°C. Then medium was replaced by Opti-MEM containing 0.1 mmol/L non-essential amino acids and 1 mmol/L sodium pyruvate. Supernatants were collected 72 h after transfection and viral stocks were stored at -80°C. The recombined lentivirus named TRPV1-Neo-RNAi(1), TRPV1-Neo-RNAi(2) and TRPV1-Neo-RNAi-empty were collected 72 hours after transfection.

3T3-L1 preadipocytes stably expressing siRNA were generated by transfection with TRPV1-Neo-RNAi(1), TRPV1-Neo-RNAi(2) and TRPV1-Neo-RNAi-empty. 3T3-L1 preadipocytes were cultured in 24-well plates to 50% confluence and 500 µL recombined lentivirus (containing 6 µg/mL polybrene) were added for transfection for 24 hours. Then cells were gently washed using PBS, fresh Opti-MEM was added, and 3T3-L1 preadipocytes were cultured for 2 days. Medium containing 800 µg/mL G418 was added to remove non-transfected cells and remaining transfected 3T3-L1 preadipocytes were further cultured. PCR was used to identify PLL3.7-Neo in transduced 3T3-L1 preadipocytes using the primers 5'-AGGAAACTCACCCTAACTG-3' and 5'-GGCTATGAACTAATGACCC-3' (Sangon Biological Engineering). Genomic DNA of transfected 3T3-L1 preadipocytes was extracted and used as template. PCR samples (total volume, 30 µL) contained 0.6 µL dNTP, 1.2 µL primer, 1 µL extracted DNA and 0.6 µl TaqDNA polymerase. PCR was started with denaturation at 95°C for 10 minutes. Then 30 cycles were performed under the following conditions: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 40 seconds. The PCR products were size fractionated on 2% agarose gels, and DNA was visualized by SYBR green staining using an imaging analyzer (Gel Doc 2000, BioRad). The expected size of the PCR product was 275 bp. The expected size of PCR products of constructed plasmids was 320 bp because of the inserted oligonucleotides. Both, RNAi-transfected and empty-control transfected 3T3-L1-preadipocytes were used for calcium measurements and for adipocyte differentiation assay.

Fluorescence measurements

Cytosolic calcium concentrations were measured in 3T3-L1-preadipocytes, 3T3-L1-preadipocytes that had been transfected with RNAi against TRPV, and adipocytes. Cells were loaded with the fluorescent dye fura2 according to previously published techniques by our group.[8,9] Cells were grown on square coverslips, washed twice with physiological saline solution (PSS, containing in mmol/L, NaCl 135, KCL 5, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 5.5, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) 10, pH 7.4 and incubated with 2 µmol/L of the cell-permeant fura2-acetoxymethylester for 60 minutes at 37°C. After loading of the cells with fura2, the experiments were continued only when a viability higher than 95% was observed using trypan blue exclusion. At the end of the loading period, the coverslips were washed twice in PSS and the fluorescence intensity of fura2-loaded cells was measured at 37°C using a spectrophotometer equipped with a thermostatically controlled cuvette holder, The light source was a 150 Watt xenon lamp with ozone self-dissociation function. The

wavelength drive motors and slit control motors were operated using the computer. The wavelength accuracy was better than ±5nm. Output signals from the monitor detector and he fluorescence detector (photomultiplier) were processed via the analog-to-digital converter. The complete intracellular hydrolysis of fura2-acetoxymethyester to fura2 was judged by the changes in the excitation and emission spectra. The fluorescence of fura2 was measured using a data-sampling interval of 1 second with alternate excitation wavelengths of 340 nm and 380 nm (bandwidth, 5nm), and the emission was collected at 510 nm (bandwidth, 5 nm). Autofluorescence was measured in similar cells which had not been loaded with fura2-acetoxymethylester and was less than 5% of the total fluorescence of fura2-loaded cells. After subtraction of autofluorescence for each wavelength, the ratio (R) of the measured fluorescence values at 340 nm and 380 nm excitation was calculated. The F340/F380 excitation ratio of resting cells remained constant during the whole experiment, indicating a stable resting calcium concentration. Leakage during the measurements was less than 4% of the total fluorescence as observed by quenching external fluorescence with 100µmol/L $MnCl_2$. Cells were stimulated with 1 µmol/L capsaicin if not indicated otherwise. Calcium influx was also determined in suspensions of human visceral adipose tissue after collagenase digestion and loading with the fluorescent dye fura2 as described above.

Immunoblotting

Immunoblottings of transient receptor potential vanilloid type-1 (TRPV1) channel, ß-actin, peroxisome proliferator-activated receptor-gamma (PPAR-γ), and fatty acid synthase from 3T3-L1 preadipocytes or adipose tissue were performed using standard techniques as reported by our group previously.[8,9] Tissue was homogenized in high-salt extraction buffer containing 50 mmol/L Tris, 1 mL NP40, 1 mL TritionX-100, 0.1 g sodium dodecyl sulfate, 150 mmol NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol ethylene glycol-bis(2-aminoethylether)-N,N',N'-tetraacetic acid. Tissue was transferred to Eppendorf tubes, and homogenized by sonication for 5 seconds. The protein supernatant was separated by centrifugation, and protein concentrations were determined with Bio-Rad protein assay reagent (Bio-Rad, Hercules CA). Proteins were separated by using 10% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes at 90 mA overnight, and immunodetected with the ECF kit (Amersham). Rabbit anti-TRPV1 antibodies were purchased from alomone labs (Jerusalem, Israel) and other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After incubation with the secondary antibodies for 1 hour, the proteins were detected by enhanced chemiluminescence and quantified using a

Gel Doc 2000 Imager (Bio-Rad). Each sample was processed at least 3 times. In-cell Western assay of TRPV1 channels human visceral adipose tissue was performed using the Odyssey infrared imaging system (Licor biosciences) as described by our group.[9] Primary rabbit anti-human TRPV1-antibodies were obtained from Alomone labs (Jerusalem, Israel) and secondary IRDye800CW-infrared fluorescent dye-conjugated goat anti-rabbit antibodies were obtained from Biomol (Hamburg, Germany). Imaging was performed at 810 nm emission with an excitation wavelength of 780 nm.

Immunohistochemistry

For immunohistochemistry isolated 3T3-L1 preadipocytes or adipocytes from visceral adipose tissue were fixed on glass slides for 10 minutes in 70 % ethanol at room temperature, washed twice in phosphate buffered saline, and fixed cells were dried on air, blocked with 1% bovine serum albumin for 1 h and then incubated with the primary antibody, TRPV1 (1:200), overnight at 4°C. Cells were then washed three times with phosphate buffered saline and then incubated for 30 minutes at 37°C with the fluorescent-dye labeled secondary antibody (IRDye800CW-infrared fluorescent dye-conjugated sheep anti-rabbit antibodies, 1:200; Santa Cruz Biotech, Inc. CA, USA; 1:1000) and analyzed with a Motic microscope. Control experiments were performed in the absence of primary antibody. Non-specific fluorescence in the absence of primary antibody was less than 3% of the fluorescence in the presence of the primary antibody.

Semiquantitative conventional polymerase chain reaction (PCR)

Expression of TRPV1 mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as internal control was measured in 3T3-L1 preadipocytes or adipose tissue after extraction of RNA by tripure isolation reagents. RNA was used to synthesize first-strand cDNA using the Reverse Transcription System (Promega, Madison WI), 4 µg of purified mRNA was reverse-transcribed with a RT mixture consisting of oligo dT (12 to 18) and 5 U AMV reverse transcriptase at 42°C for 60 minutes, followed by heating to 99°C for 5 minutes. The single stranded cDNA was amplified by PCR using taq DNA polymerase (Sangon Biological Engineering, Shanghai, China). PCR reaction was performed using 3 µL of cDNA, 3 µL of PCR buffer 10x, 10mmol/l dNTP, 0.6 µL primers, and 2.5 IU taq DNA polymerase in a total reaction volume of 30µL. The sense and antisense primers for coding regions of TRPV1 or GAPDH genes were the following: TRPV1 (Reference Sequence (RefSeq) database accession number: NM_001001445), forward, 5'-

TGCCCTATCATCACCGTCAG-3'; reverse, 5'-GTGTTGCCCACCGAATCCC-3'; expected size, 644bp; GAPDH (RefSeq BC095932); forward, 5'-ACCTCAACTACATGGTCTAC-3′ reverse, 5'-TTGTCATTGAGAGCAATGCC-3′; expected size, 802bp. The reaction was initiated at 94°C for 10 minutes and followed by 35 cycles of denaturation at 94°C for 90 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 60 seconds. The reaction was achieved with a final extension at 72°C for 7 minutes. Each sample was run in parallel with the housekeeping gene. Control experiments in which reverse transcriptase was omitted were performed in order to exclude the possibility that the signal detected could come from the genomic DNA. 10 μL of the PCR products were size fractionated on agarose gel (1.5%) and quantified using a Gel Doc 2000 Imager (Bio-Rad).

Quantitative real-time PCR

In visceral adipose tissue and in subcutaneous fat from humans the expression of TRPV1 mRNA and GAPDH mRNA as internal control was quantified using quantitative real-time PCR (LightCycler, Roche Diagnostics, Mannheim, Germany). RNA was extracted and first-strand cDNA was synthesized as described above. Gene specific PCR primers for human TRPV1 and the housekeeping gene GAPDH were designed using Lasergene7 (DNA-Star, Madison, Wisconsin). To avoid the amplification of genomic DNA, primers were chosen in different exons. The primers for real-time PCR were the following: human TRPV1 (RefSeq NM_080704); forward, 5'-CTCCCTGCCGTCTGAGTCCA-3'; reverse, 5'-AGTGTCTGCCTGAAACTCTGCTT-3'; expected size, 548bp. human GAPDH (RefSeq NM_002046); forward, 5'-AACTGCTTAGCACCCCTGGC-3′; reverse, 5'-ATGACCTTGCCCACAGCCTT-3′; expected size, 200bp. The TRPV1 and the GAPDH housekeeping gene were amplified in parallel. Quantitative real-time PCR was performed using 2 μL of single stranded cDNA which was added to a final volume of 20 μL, which contained 4 μL of LightCycler-Fast Start DNA SYBR Green I mix (Roche Diagnostics), and 500 nmol/L of each primer. The reaction was initiated at 95°C for 10 minutes, and followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing for 10 seconds at 58°C for TRPV1 or at 54°C for GAPDH, and extension at 72°C for 15 seconds. Fluorescent data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65°C to 95°C with a heating rate of 0.1°C per second with a continuous fluorescence acquisition was made. Data were recorded on an LightCycler 2.0 Instrument (Roche Diagnostics) and cycle thresholds (crossing points, Cp) values for each reaction were determined using LightCycler Software Version 4.0 (Roche Diagnostics). Calibration curves

were generated from serial dilutions of cDNAs for both TRPV1 gene and housekeeping gene GAPDH, respectively. Relations (plots of Cp versus the log of dilution) were linear and showed a correlation coefficient of 0.99. The efficiency was 2.091 for TRPV1 and the efficiency was 1.986 for GAPDH. Each cDNA of four independent experiments was amplified in duplicate and Cp values were averaged from each duplicate. To account for differences in RNA loading TRPV1 was expressed relative to GAPDH housekeeping gene. Normalized ratios of TRPV1 expression were calculated according to the manufacturers recommendations including efficiency correction and calibrator normalization.

Animals

All mice (db/db mice, ob/ob mice, C57BL/6 wild-type mice, and TRPV1 knock-out mice) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). For feeding experiments mice were obtained from an in-house breeding colony based on a C57BL/6 wild-type genetic background (The Jackson Laboratory). Male mice were randomized into four groups; one group received standard laboratory chow, one group received high-fat diet, one group received standard laboratory chow plus 0.01% capsaicin, one group received high-fat diet plus 0.01% capsaicin. Hence, the approximate capsaicin intake was 1.64 μmol/day per mouse. Assuming a bioavailability of 1% the capsaicin intake produces a plasma concentration of more than 10 μmol/L in mice. The high-fat diet supplied 49% of the calories as fat and 30% of the calories as carbohydrate. The standard laboratory chow diet provided 10% of the calories as fat and 68% as carbohydrate. Mice were housed in a pathogen-free animal facility and allowed water and food ad libitum. Food intake was controlled every day. All animals were subject to controlled temperature (22 ± 1 °C) and lighting (lights on 06.00 to 18.00). Serum triglycerides were analyzed using routine techniques. Animal treatment and care was provided in accordance with institutional guidelines. All of the experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Determination of adipocyte size

Adipocyte size was determined in tissue according to established techniques.[10] Tissue was fixed in 10% formaldehyde/phosphate buffered saline and embedded in paraffin. Sections were stained with hematoxylin and eosin and studied under x400 magnification compare adipocytes size using a light microscope (Motic Digital Microscope B5 Professional, Xiemen,

China) and photomicrographs were obtained. The adipocytes diameter and area were analyzed using IPLab software (Scanalytics Inc, Fairfax, VA).

Patients

Subjects were classified obese if waist circumference was more than 90 cm according to the Asian criteria of the WHO Regional Office for the Western Pacific / International Association for the Study of Obesity/International Obesity Task Force.[11,12] Age, body mass index, waist circumference, and triglycerides were obtained. TRPV1 channel expression was evaluated in visceral adipose tissue and subcutaneous fat which was obtained during regular scheduled cholecystectomy. Cholecystektomy had to be performed because of symptomatic gallstones. Visceral fat tissue was also obtained from subjects scheduled for an urological procedure. The protocol was approved by the local Ethics Committee. All patients gave written informed consent.

Statistics

All values reported are mean ± SEM. Comparisons between groups were analyzed using Student t-test or one-way ANOVA with Bonferroni's multiple comparison post hoc test as appropriate (GraphPad Prism; LaJolla CA). Two-sided p values below 0.05 were considered to indicate statistical significance.

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