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Full-length article

Downregulation of STEAP4, a highly-expressed TNF-α-inducible gene in adipose tissue, is associated with obesity in humans[1]

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Key words

six-transmembrane epithelial antigen of the prostate 4; obesity; tumor necrosis factor-α; adipose tissuess

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Abstract

Aim: To determine the relationship between six-transmembrane epithelial antigen of the prostate 4 (STEAP4) expression and obesity. **Methods:** RT–PCR and immunoblot analyses were performed to determine the differential expressions of STEAP4 mRNA and protein, respectively, in human omental adipose tissue from obese patients and normal weight controls. The expression pattern of STEAP4 mRNA in various human tissues was determined by RT–PCR. The subcellular localization of the STEAP4 protein in human adipose tissue was confirmed by immunohistochemistry. Finally, we confirmed that cultured human omental adipose tissue undergoes TNF-α-mediated regulation of the STEAP4 expression. **Results:** STEAP4 mRNA and protein levels were downregulated in omental adipose tissue from obese patients relative to normal controls. The STEAP4 expression was most abundant in human adipose tissue. An immunohistochemical analysis confirmed that STEAP4 was associated with the plasma membrane of adipocytes. The STEAP4 expression was induced by TNF-α in a dose-dependent manner in human adipose tissue. **Conclusion:** STEAP4 was abundantly expressed in human adipose tissue, and the STEAP4 expression was significantly downregulated in obese patients. STEAP4 localized to the plasma membrane of adipocytes, and the STEAP4 expression was induced by TNF-α in adipose tissue. These data suggest that STEAP4 may play a significant role in the development of human obesity.

Introduction

Obesity is a multifactorial disease resulted from interactions between susceptibility genes and environmental factors. Furthermore, obesity is an important risk factor for other diseases, including type 2 diabetes, hypertension, hyperlipidemia, and cardiac infarction[1-3]. During the development of obesity, adipose tissue plays a key role in energy homeostasis by regulating the balance between energy storage and energy consumption in response to nutritional status[1]. Therefore, the identification and functional characterization of genes whose expression is differentially regulated in obese patients relative to normal weight controls may provide new insights into the molecular mechanisms underlying obesity-associated pathogenesis.

In a previous study, we performed suppression subtractive hybridization[4-5] using omental adipose tissue from obese patients and normal weight controls. We identified 426 differentially-expressed genes in adipose tissue from obese patients. In total, 216 genes were upregulated and 210 genes were downregulated[6]. Among these genes, we found that the expression of six-transmembrane epithelial antigen of the prostate (STEAP) 4, a member of the STEAP protein family, was significantly downregulated in the obese patients, suggesting that STEAP4 may be associated with obesity. Although a previous study had shown an association between STEAP4 overexpression and human prostate cancer[7], a link between human STEAP4 and obesity had not been reported. Studies of the mouse homolog of STEAP4, six-transmem-

brane protein of prostate (STAMP) 2 [previously called tumor necrosis factor (TNF)-α-induced adipose-related protein], which shares 90% amino acid identity with human STEAP4, showed TNF-α-mediated STAMP2 induction and adipose conversion[8]. Recently, Wellen *et al* identified STAMP2 as a critical modulator of inflammation and nutrition, suggesting a potential role for STEAP4 in human obesity[9].

In the present study, we determined the relationship between human STEAP4 and obesity. We analyzed: the differential expression of STEAP4 in adipose tissue from obese patients relative to normal controls, the STEAP4 expression in a panel of human tissues, the subcellular localization of STEAP4 in human adipose tissue, and the TNF-α-mediated induction of STEAP4 in human adipose tissue. Taken together, these findings are in agreement with previous studies of mouse STAMP2 and suggest that STEAP4 is likely to play a significant role in the development of human obesity.

Materials and methods

Antibody preparation Rabbit polyclonal antiserum was generated against an N-terminal peptide of STEAP4 (AEYLAHLVPGAHVVKAC), coupled to bovine serum albumin and subjected to antigen affinity purification (GL Biochem, Shanghai, China).

Analysis of the differential expressions of STEAP4 mRNA and protein All human omental adipose tissues were obtained from male patients undergoing abdominal surgery for acute simple appendicitis. Patients were assigned to the normal weight group or obese group according to their body mass index (BMI) [10], which was defined as weight in kilograms divided by the square of height in meters. When patients' BMI =30 kg/m^2, they were assigned to the obese group (n=6; BMI 30.3±0.3; age 50.6±9.2); when BMI scores fell between 18 and 25 kg/m^2, the patients were assigned to the normal group (n=6; BMI 22.2±1.7; age 47.1±11.7). Written consent was obtained from each patient, and the experiments were conducted according to the Declaration of Helsinki.

The differential expression of STEAP4 mRNA was analyzed using RT–PCR. Total RNA from the omental adipose tissues of obese and normal weight patients was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA samples (200 ng) were subjected to RT–PCR using random primers with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA), and an aliquot (10%) of the resulting cDNA was amplified using GAPDH and STEAP4-specific primers. The primer sequences for STEAP4 and GAPDH were as follows: STEAP4, 5'-CGAAACTTC CCTCTACCCG-3' (sense) and 5'-ACACAAACACCTGCCGACTT-3' (antisense); GAPDH, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' (sense) and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (antisense). After denaturation at 94 °C for 3 min, 34 cycles of PCR amplification were performed (30 s at 94 °C, 30 s at 58 °C, and 40 s at 72°C). A final extension step was performed at 72 °C for 7 min. Finally, 5 μL of the PCR product was loaded on a 1.5% agarose gel.

The STEAP4 protein expression was analyzed by immunoblotting, as described previously[6]. Briefly, approximately 100 mg of fresh or frozen adipose tissue was resuspended in 1 mL lysis buffer (50 mmol/L Tris-HCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, and 1 mmol/L EDTA at pH 7.4) and homogenized using a polytron homogenizer at 4 °C. The lysate supernatant was collected after centrifugation at 20 670×g for 30 min at 4 °C. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA), and 30 μg of total protein was loaded in each lane of a 10% polyacrylamide gel, followed by SDS–PAGE. Prestained protein standards (Fermentas, Hanover, MD, USA) were used as molecular weight markers. The separated proteins were electrophoretically transferred to a nitrocellulose membrane filter (Whatman, GmbH, Dassel, Germany), and the membrane was blocked with 5% dried milk for 2 h at room temperature. Subsequently, the membranes were incubated at 4 °C overnight in a primary antibody (polyclonal rabbit anti-STEAP4 antibody or monoclonal mouse anti-GAPDH antibody; KangChen Bio-tech, Shanghai, China) at an appropriate dilution, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. STEAP4 and GAPDH bands were detected using enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA) and hyperfilm exposure.

Analysis of STEAP4 expression in a panel of human tissues RT–PCR was performed using specific primer pairs to amplify human STEAP4 and GAPDH (primer sequences given earlier) in a panel of human tissues. We purchased the human multiple tissue cDNA (MTC) panel I (Clontech, Mountain View, California, USA). The adipose tissue cDNA sample was prepared manually from normal weight human omental adipose tissue obtained with prior consent during abdominal surgery for appendicitis. The human normal testis and spleen cDNA samples were purchased from Bio/Biotech (Shanghai, China). All PCR reactions were performed using the following protocol: denaturation at 94 °C for 30 s, followed by 35 amplification cycles (30 s at 94 °C, 30 s at 60 °C,

and 40 s at 72 °C), and a final extension at 72 °C for 5 min. Negative controls (no cDNA template) were included with both the GAPDH- and STEAP4-specific primer reactions. Gel analysis was performed using aliquots removed at cycle 22 for GAPDH and at cycle 34 for STEAP4; 5 μL of the amplification product was loaded on a 1.5% agarose gel.

Immunohistochemistry Human omental adipose tissues were fixed at 4 °C overnight with 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.2 and embedded in paraffin. Next, 5 μm-thick sections were cut and mounted onto microscope slides. The sections were deparaffinized with xylene and hydrated in graded ethanol. After deparaffinization, the sections were subjected to antigen retrieval in 0.01 mol/L sodium citrate at pH 6.0 at 100 °C for 10 min in a microwave oven. They were then incubated at 4 °C overnight with a polyclonal anti-STEAP4 antibody (1:100 dilution). After washing with phosphate-buffered saline, the sections were treated with a goat antirabbit HRP-conjugated secondary antibody (1:100 dilution) at 37 °C for 1 h. The primary antibody was detected using a diaminobenzidine (DAB) kit (ZhongShan Golden Bridge Biotechnology, Beijing, China) according to the manufacturer's instructions. The sections were counterstained with hematoxylin, observed under a light microscope, and photographed. Negative controls were obtained for each section by omitting the primary antibody and by using pre-immune serum instead of the primary antiserum.

TNF-α induction Human omental adipose tissues were obtained from normal weight patients during abdominal surgery for appendicitis. The samples were processed as described previously[11], and the minced adipose tissue fragments were placed in serum-free M199 (Invitrogen, USA) containing 25 mmol/L HEPES with or without increasing concentrations of TNF-α (5, 50, and 100 μg/L; Sigma, St Louis, MO, USA). Cultures were maintained for 48 h. At the end of culture, the tissues were rapidly washed in saline, frozen in liquid nitrogen, and stored at –80 °C until the analysis. STEAP4 mRNA and protein expressions were determined by RT–PCR and an immunoblot analysis, respectively, as described earlier.

Statistical analysis All data are expressed as mean±SEM. Statistical analysis was performed using the paired Student's *t*-test of the SPSS 10.0 statistical software package (SPSS, Chicago, IL, USA). The threshold of significance was defined as $P<0.05$.

Results

Differential expression of STEAP4 in adipose tissue from obese patients and normal weight controls STEAP4 mRNA and protein expressions in human omental adipose tissues from obese patients and normal weight control were determined by RT–PCR and an immunoblot analysis, respectively. As shown in Figure 1, STEAP4 mRNA levels were lower in adipose tissue from obese patients relative to normal weight controls. The immunoblot analysis showed approximately a 52 kDa band that corresponded to the predicted size of STEAP4 (data not shown). Moreover, STEAP4 protein levels were also lower in the adipose tissue from obese patients relative to the normal weight controls (Figure 2).

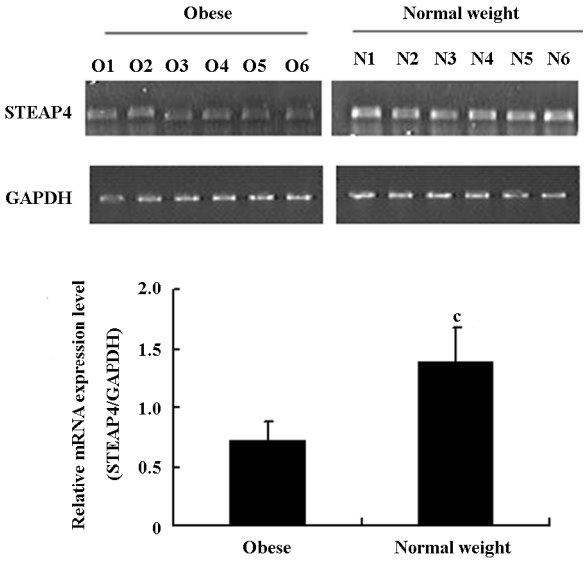

Figure 1. RT–PCR analysis shows the differential expression of STEAP4 mRNA in omental adipose tissue from obese patients relative to normal weight controls. STEAP4 mRNA levels were quantified and normalized to GAPDH mRNA levels. STEAP4 mRNA levels were significantly lower in obese patients relative to normal weight controls. $n=6$. $^cP<0.01$ *vs* normal weight controls. O, obese; N, normal weight.

STEAP4 mRNA expression pattern in a panel of human tissues We performed RT–PCR using a panel of human cDNA samples to determine the tissue distribution of STEAP4 mRNA; this study included 11 human adult tissues. Figure 3 shows that among the human tissues tested, adipose tissue showed the highest levels of the STEAP4 expression, followed by placenta and lung. Lower levels of STEAP4 transcripts were detected in the skeletal muscle and kidney, whereas STEAP4 mRNA was undetectable in the spleen and brain.

STEAP4 protein localizes to the plasma membrane of adipocytes Because the subcellular localization of human STEAP4 protein in adipose tissue may provide key insights regarding STEAP4 function, we performed immunohis-

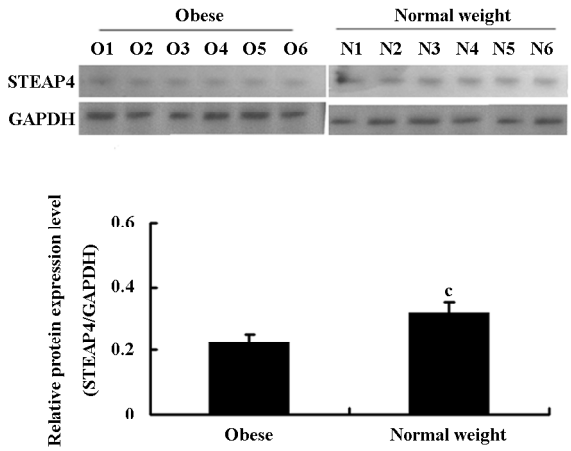

Figure 2. Downregulation of STEAP4 protein levels in obese patients relative to normal weight controls. STEAP4 protein levels in omental adipose tissue were determined by immunoblot analysis. STEAP4 protein levels were downregulated in obese patients relative to normal weight controls. n=6. ^cP<0.01 *vs* normal weight controls. O, obese; N, normal weight.

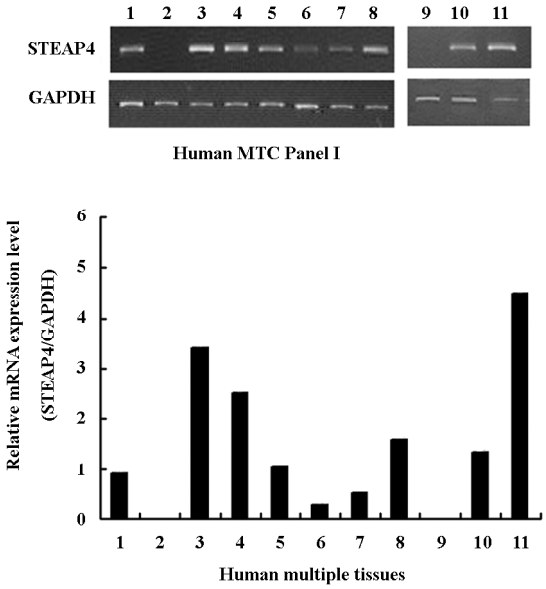

Figure 3. Analysis of STEAP4 expression in a panel of human tissues. STEAP4 mRNA levels were analyzed in a panel of human tissues using human MTC panel I and other human cDNA samples. STEAP4 mRNA levels were normalized to GAPDH for each tissue. Lane 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, testis; and 11, omental adipose tissue.

tochemical analyses of human adipose tissue using anti-STEAP4 antibodies and HRP-conjugated secondary antibodies. Figure 4 shows that the STEAP4 protein localized to the plasma membrane of adipocytes, suggesting that

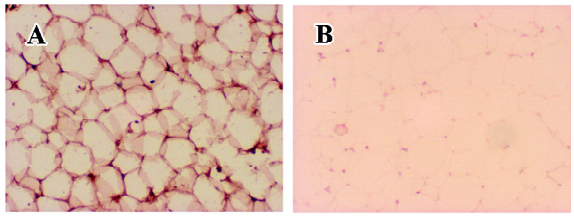

Figure 4. Immunohistochemical analysis of the STEAP4 expression in human adipose tissue. Human adipose tissue samples were fixed in 4% paraformaldehyde at 4 °C overnight and were embedded in paraffin. Sections were incubated with rabbit polyclonal anti-STEAP4 antibodies, followed by incubation with a goat antirabbit HRP-conjugated secondary antibody. Primary antibody was detected using a DAB kit, and sections were counterstained with hematoxylin. (A) STEAP4; (B) negative control. Magnification ×100.

STEAP4 might be a membrane-associated protein.

TNF-α-induced modulation of STEAP4 mRNA and protein expressions To determine whether the STEAP4 gene expression could be induced by TNF-α, we examined the STEAP4 expression in primary cultures of adipose tissue treated with increasing levels of TNF-α. As shown in Figure 5, TNF-α led to a dose-dependent increase in the STEAP4 mRNA expression after 48 h of treatment. This effect was undetectable at 5 μg/L TNF-α, and reached the maximum at 50 μg/L; a half-maximal response was obtained at 100 μg/L. Similarly, the STEAP4 protein expression also increased in response to TNF-α treatment. The treatment of cultured adipose tissue for 48 h with 50 μg/L TNF-α led to a stronger increase in the STEAP4 protein expression than that induced by 100 μg/L TNF-α; this effect was undetectable in the presence of 5 μg/L TNF-α (Figure 6).

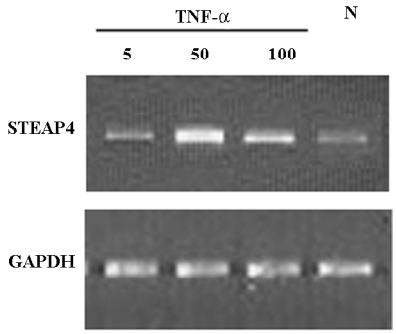

Figure 5. TNF-α-mediated induction of the STEAP4 mRNA expression in cultured adipose tissue. Cultured human adipose tissues were treated with increasing concentrations of TNF-α for 48 h. Total RNA was extracted from each sample, followed by RT–PCR analysis. Five microliters of PCR products were subjected to 1.5% agarose gel electrophoresis, and the resulting bands were visualized using ethidium bromide staining (N, without TNF-α; 5: 5 μg/L TNF-α; 50, 50 μg/L TNF-α; 100, 100 μg/L TNF-α).

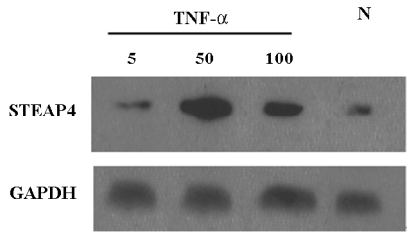

Figure 6. TNF-α-mediated induction of the STEAP4 protein expression in cultured adipose tissue. Cultured human adipose tissues were treated with increasing concentrations of TNF-α for 48 h. Protein lysates were subjected to SDS–PAGE, followed by immunoblot analysis using anti-STEAP4 and anti-GAPDH antibodies (N, without TNF-α; 5, 5 μg/L TNF-α; 50, 50 μg/L TNF-α; 100, 100 μg/L TNF-α).

Discussion

Obesity is a multifactorial disease resulted from interactions between susceptibility genes and environmental factors. The obesity gene map reveals that putative loci affecting obesity-related phenotypes are found on all autosomes and on the Y chromosome. The number of genes, markers, and chromosomal regions that have thus far been associated with or linked to obesity phenotypes has reached 200 and continues to increase[12].

The mouse STAMP2 gene has been reported recently to play a role in the coordinated regulation of nutrient and inflammatory responses in adipose tissue[9]. However, a role for human STEAP4, the homolog of mouse STAMP2, in obesity has not been reported. In the present study, we found that the STEAP4 expression was downregulated in adipose tissue from obese human patients. Although our case number is only 6, STEAP4 downregulation occurred consistently at both the mRNA and protein levels. These findings suggest a close relationship between STEAP4 downregulation and obesity in humans. Moreover, we found that STEAP4 exhibited a tissue-specific pattern of expression pattern, and that STEAP4 is most highly expressed in human adipose tissue. Korkmaz *et al* showed that STEAP4 was expressed most highly in the placenta, lung, heart, and prostate among 16 different human tissues[7]; however, their study did not examine the STEAP4 expression levels in adipose tissue. We found that the STEAP4 expression was highest in adipose tissue, followed by the placenta, lung, and heart. Taken together, our results strongly support an active role for STEAP4 in adipose tissue and suggest that STEAP4 might contribute to obesity.

To explore further the role of STEAP4 in human adipose tissue, we confirmed that human STEAP4 exhibited a plasma membrane-associated pattern of localization in human adipocyte tissue. Furthermore, the STEAP4 expression was induced in a dose-dependent manner by TNF-α in cultured human adipose tissue. TNF-α exerts a well-established, key regulatory role in obesity and in obesity-related insulin resistance (IR)[13]; TNF-α also markedly alters adipose tissue development and metabolism[14-17]. Overwhelming evidence suggests that TNF-α could regulate the expression of many obesity-related genes, including resistin, leptin, visfatin, adiponectin[18-20], and of particular interest, the mouse homolog of human STEAP4, STAMP2[8]. Therefore, we hypothesized that STEAP4 might also be regulated by TNF-α, in a manner similar to that of STAMP2. This would implicate STEAP4 as a key mediator of the physiological or pathological effects of TNF-α, which may modulate several adipocyte-associated functions, including differentiation, lipolysis, lipogenesis, insulin sensitivity, and apoptosis. In the present study, we demonstrated that TNF-α treatment led to a dose-dependent induction of the STEAP4 expression in cultured human adipose tissue, at both the mRNA and protein levels. However, it seemed to be contradictory that in obese patients with high serum TNF-α level, the STEAP4 expression level was lower than the normal controls. We considered although TNF-α was an important regulator of the STEAP4 expression *in vitro*, the STEAP4 expression may also be modulated by some other factors *in vivo*. The similar phenomenon was observed in the study of resistin, which is abundantly expressed in obesity but its expression could be suppressed by TNF-α *in vitro*[18, 21]. Until now, the relationship between cytokines, such as TNF-α with the STEAP4 expression, remains an unresolved question. This work may make it a little clearer.

The identification of a STEAP4 N-terminal domain with associated nicotinamide-adenine dinucleotide phosphate (NADP) oxidoreductase coenzyme activity[7] provides an interesting clue about potential STEAP4-associated biochemical functions. Taken together with previous studies, including the discovery that an NADPH-dependent H_2O_2-generating system was associated with human and rodent adipocyte plasma membranes[22, 23] and that reactive oxygen species (ROS) played a vital role in obesity and in obesity-related IR[24], these findings suggest that STEAP4 might be involved in the ROS-related pathological pathway and may eventually contribute to the development of obesity and obesity-related IR.

Our studies were based on recent findings by Wellen *et al* who systematically identified functional links between STAMP2 and obesity in mice. Our study focused on STEAP4, the human homolog of STAMP2. In conclusion, STEAP4 was abundantly expressed in human adipose tissue and that the STEAP4 expression was significantly downregulated in

obese patients. Furthermore, STEAP4 underwent a dose-dependent induction in response to TNF-α treatment. Collectively, these findings provide new insights into how the STEAP4 and TNF-α pathways may contribute to the development of obesity and obesity-related IR in humans.

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