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Monoclonal antibody to six transmembrane epithelial antigen of prostate-4 influences insulin sensitivity by attenuating phosphorylation of P13K (P85) and Akt: Possible mitochondrial mechanism

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Abstract We examined the effects of anti-six-transmembrane epithelial antigen of the prostate-4 (STEAP4) antibodies on glucose transport in mature adipocytes and determined the mechanism of insulin resistance in obesity. Western blotting was performed to determine STEAP4 expression, to assess translocation of insulin-sensitive glucose transporter 4 (GLUT4), and to measure phosphorylation and total protein content of insulin-signaling proteins. Confocal laser microscopy and flow cytometry were used to detect intracellular reactive oxygen species (ROS) and fluctuations in mitochondrial membrane potential ($\Delta\Psi$). ATP production was measured by using a luciferase-based luminescence assay kit. After the application of anti-STEAP4 antibodies at 0.002 mg/mL, adipocytes exhibited reduced insulin-stimulated glucose transport by attenuating the phosphorylation of IRS-1, PI3K (p85), and Akt. The antibodies also potentially increase the level of ROS and decrease cellular ATP production and $\Delta\Psi$. In conclusion, (i) STEAP4 regulates the function of IRS-1, PI3K, and Akt and decreases insulin-induced GLUT4 translocation and glucose uptake; (ii) ROS-related mitochondrial dysfunction may be related to a reduced IRS-1 correlation with the PI3K signaling pathway, leading to insulin resistance. These observations highlight the potential role of STEAP4 in glucose homeostasis and possibly in the pathophysiology of type 2 diabetes related to obesity and may provide new insights into the mechanisms of insulin resistance in obesity.

Keywords STEAP4 antibody · Obesity · Insulin sensitivity · Insulin receptor substrate-1 · Phosphatidylinositol-3-kinase

Abbreviations

STEAP4	six-transmembrane epithelial antigen of the prostate 4
GLUT4	glucose transporter 4
IR	insulin receptor
IRS	insulin receptor substrate
PI3K	phosphatidylinositol-3-kinase
ERK1/2	extracellular signal-regulated kinases 1 and 2
JNK	c-Jun N-terminal kinase
PM	Plasma membrane
MAPK	mitogen-activated protein kinase
ROS	reactive oxygen species

Introduction

The six-transmembrane epithelial antigen of the prostate 4 (STEAP4; also referred to as STAMP2 and

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TIARP), a member of the six-transmembrane proteins family, has attracted considerable interest in obesity and energy metabolism research (Alessi et al. 1996; Andjelković et al. 1996; Andjelković et al. 1997; Arner et al. 2008). Biochemical and secondary structural analyses suggest that this protein could potentially function as a channel, receptor, or transporter protein; however, its true function has not yet been clarified. In STAMP2−/− mice, the visceral adipose tissue exhibits overt inflammation, thereby manifesting insulin resistance, glucose intolerance, mild hyperglycemia, dyslipidemia, and fatty liver disease (Andjelković et al. 1996). Our previous data showed that the human *STEAP4* gene is highly expressed in omental adipose tissue and participates in obesity development (Boura-Halfon and Zick 2008). Recently, we also found that over-expression of the *STEAP4* gene promoted insulin-stimulated glucose uptake in mature human adipocytes (Challita-Eid et al. 2007). Taken together, the data indicate that this gene may influence insulin sensitivity; however, the mechanisms underlying insulin sensitivity are only partially understood. Therefore, additional research on the STEAP4 function is necessary and will aid in our efforts to study the STEAP4 mechanism and how it influences insulin sensitivity.

Monoclonal antibodies have been used widely in clinical and experimental studies (Chen et al. 2010; Corvera and Czech 1998; Dani et al. 2010a). In addition, immunohistochemical analysis has confirmed that STEAP4 was associated with adipocyte plasma membranes (Boura-Halfon and Zick 2008), and therefore, provides a basis for our decision to use antibodies against human adipocytes. In recent studies, the generated monoclonal antibodies had bound to the extracellular domains of human STEAP4, suggesting that these antibodies may function to promote or inhibit STEAP4 function (Dani et al. 2010b). In this study, we examined the effects of anti-STEAP4 antibodies on glucose transport in mature adipocytes and determined the molecular events underlying these effects. The results suggested that upon anti-STEAP4 antibody exposure at 0.002 mg/mL, adipocytes reduced insulin-stimulated glucose transport (Ducluzeau et al. 2002) by attenuating phosphorylation of the insulin receptor substrate (IRS)-1, phosphatidylinositol-3-kinase (PI3K) p85, and Akt. Meanwhile, it also increases the intracellular reactive oxygen species (ROS), while reducing cellular ATP production and mitochondrial $\Delta\Psi$. Thus, the mechanism by which STEAP4 affects insulin sensitivity may lie in the PI3K-protein kinase B/Akt (PKB) pathway, and the ROS-related mitochondrial dysfunction may be related to a reduced IRS-1 correlation with the PI3K signaling pathway, leading to insulin resistance.

Materials and methods

Antibodies

Mouse monoclonal antibodies against STEAP4 were purchased from Lifespan Biosciences (LifeSpan Biosciences, Seattle, WA, USA). Primary polyclonal glucose transporter 4 (GLUT4) antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (1:500; Santa Cruz, CA, USA). Anti-phospho-insulin receptor (IR) beta (Tyr1146), anti-IR beta, phosphatidylinositol-3-kinase [(PI3k) p85α/γ (Y463)], and anti-IRS-1 were purchased from Cell Signaling Technology (Danvers, MA, USA). The phospho-specific polyclonal antibody against IRS-1 (Tyr612) was obtained from Biosource (1:500; Camarillo, CA, USA). Antibodies against Akt, ERK1/2, and the phosphorylated forms of these proteins were obtained from Bioworld (1:500; Georgia, USA). Antibodies against p38, JNK, and the phosphorylated forms of these proteins were also obtained from Bioworld (1:500; Georgia, USA).

Cell culture and treatment

Human pre-adipocytes (Sciencell Research Laboratories, San Diego, CA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in 5% CO_2. To induce differentiation, confluent human pre-adipocytes (D0) were cultured in serum-free DMEM that contained 50 nM insulin, 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 100 μM rosiglitazone. The medium was changed every 2 days for the first 4 days. Thereafter, the medium was replaced with serum-free DMEM that contained 50 nM insulin and 100 nM dexamethasone, and the medium was changed every 2 days until lipid droplets had accumulated (days 14–17). Fat accumulation was assessed by staining formalin-fixed cells with Oil Red O.

Immunoblotting

Human pre-adipocytes were induced to differentiate as described above. On day 15, cells were exposed to antibodies against STEAP4 for 24 h, then serum-starved for 3 h, and finally incubated with or without 100 nmol/L insulin. Total proteins and phosphorylated proteins were extracted as described previously (Garcia-Hernandez Mde et al. 2007). Plasma membrane (PM) proteins were extracted using Eukaryotic Membrane Protein Extraction Reagent (Merck KGaA, Darmstadt, Germany). Protein

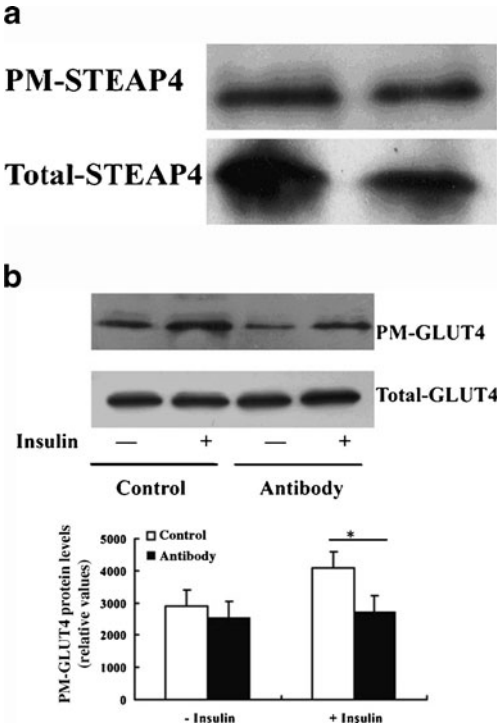

Fig. 1 **a** Membrane proteins and total proteins expression of STEAP4. **b** Effects of anti-STEAP4 antibodies on baseline and insulin-stimulated GLUT4 translocation

levels were quantified with the bicin chonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the loaded proteins (40 μg/lane) were electrophoretically transferred onto a nitrocellulose membrane (Whatman, London, UK). After blocking with Tris-buffered saline Tween-20 [TBST: 0.14 mol/L NaCl, 0.02 mol/L Tris base (pH 7.6), and 0.1% Tween] that contained 3% bovine serum albumin (BSA) for 1 h at room temperature, the membrane was hybridized with primary antibodies at the appropriate dilutions overnight at 4 °C. The membrane was washed 5 times with TBST for 5 min. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed with TBST, and developed with the enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA)

Table 1 PM-STEAP4 and total-STEAP4 protein levels ($\bar{x}\pm s$)

groups	Number of samples	Protein levels (relative values)
PM-STEAP4	6	1993.833±86.92621
Total-STEAP4	6	4833.333±216.0247

Table 2 PM-GLUT4 Protein levels ($\bar{x}\pm s$)

groups	Number of samples	Protein levels (relative values)
Control - insulin	6	2551.6667±178.93202
Control+insulin	6	4167.1667±162.82104
Antibody - insulin	6	2573.3333±146.37850
Antibody+insulin	6	2823.3333±163.53477

Confocal laser microscopy and flow cytometry

The H2-DCFDA probe (Sigma, St. Louis, MO, USA) was used to estimate intracellular ROS levels (Giovannone et al. 2000). On day 15 of differentiation, the human adipocytes were incubated with anti-STEAP4 antibodies for 24 h, incubated with 5 μM H2-DCFDA for 30 min at 37 °C, and then washed 3 times with pre-warmed PBS. The spectral characteristics of the harvested cells were analyzed by FACS (excitation at 488 nm, emission at 525 nm) and the images were captured using a fluorescence microscope.

Adipocytes were incubated for 30 min at 37 °C with 150 nM Mitotracker, a red mitochondria-specific cationic fluorescent dye (Molecular Probes, Invitrogen) that differentially accumulates in the mitochondria based on the membrane potential. Following incubation, the cells were washed 3 times with pre-warmed PBS. The dye cannot exit after it enters the mitochondria, regardless of the mitochondrial membrane potential. The spectral characteristics of the harvested cells were analyzed by FACS (excitation at 579 nm, emission at 644 nm) and the images were captured on a fluorescence microscope. The fluorescent intensity reflects the mitochondrial membrane potential.

ATP production

The pre-adipocytes were induced to differentiate as described above. On day 15, the adipocytes were incubated with antibody against STEAP4 for 24 h and then the adipocyte ATP content was measured by a luciferase-based luminescence assay kit (Biyuntian, Nantong, China). Briefly, adipocytes were homogenized in ice-cold ATP-releasing buffer and an ATP standard was used to determine ATP concentrations with a single-tube luminometer (Turner Biosystems, CA, USA). The luminescence then was normalized by protein concentration.

Electron microscopy

Adipocytes were exposed to either anti-STEAP4 antibody or media alone for 24 h. Next, the cells were digested with

trypsin, washed in fresh PBS (pH 7.4), and fixed in 2.5% glutaraldehyde and 4% paraformaldehyde buffer. Cells were washed with 0.1 M cacodylate buffer, post-fixed for 1 h in 1% osmium tetroxide and 1.5% potassium ferrocyanide buffer, washed with water, stained for 30 min with 1% aqueous uranyl acetate, and then dehydrated with a graded series of ethanol up to 100%. The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada), ultrathin sections (60 nm) were cut using a Reichert Ultracut-S microtome, the sections were placed on copper grids stained with uranyl acetate and lead citrate, and finally examined on a JEOL JEM-1010 transmission electron microscope at an accelerating voltage of 80 kV.

Statistical analysis

Each experiment was performed at least 3 times. All values are presented as the means ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t* test. The threshold of statistical significance was defined as $P<0.05$.

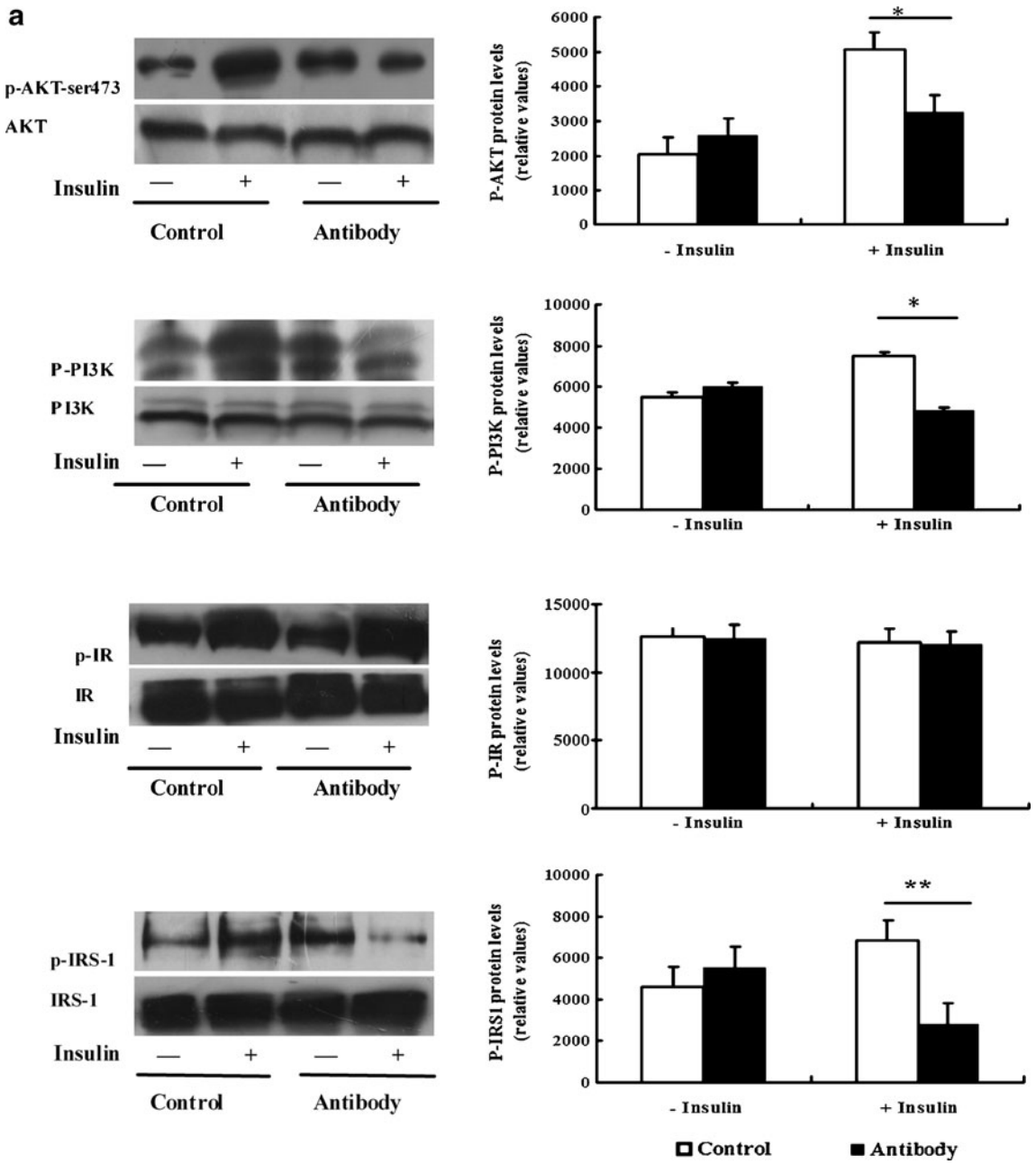

Fig. 2 **a** Effects of anti-STEAP4 antibodies on protein expression and insulin-stimulated phosphorylation of PI3K insulin signaling molecules. **b** Effects of anti-STEAP4 antibodies on protein expression and insulin-stimulated phosphorylation of MAPK insulin signaling molecules

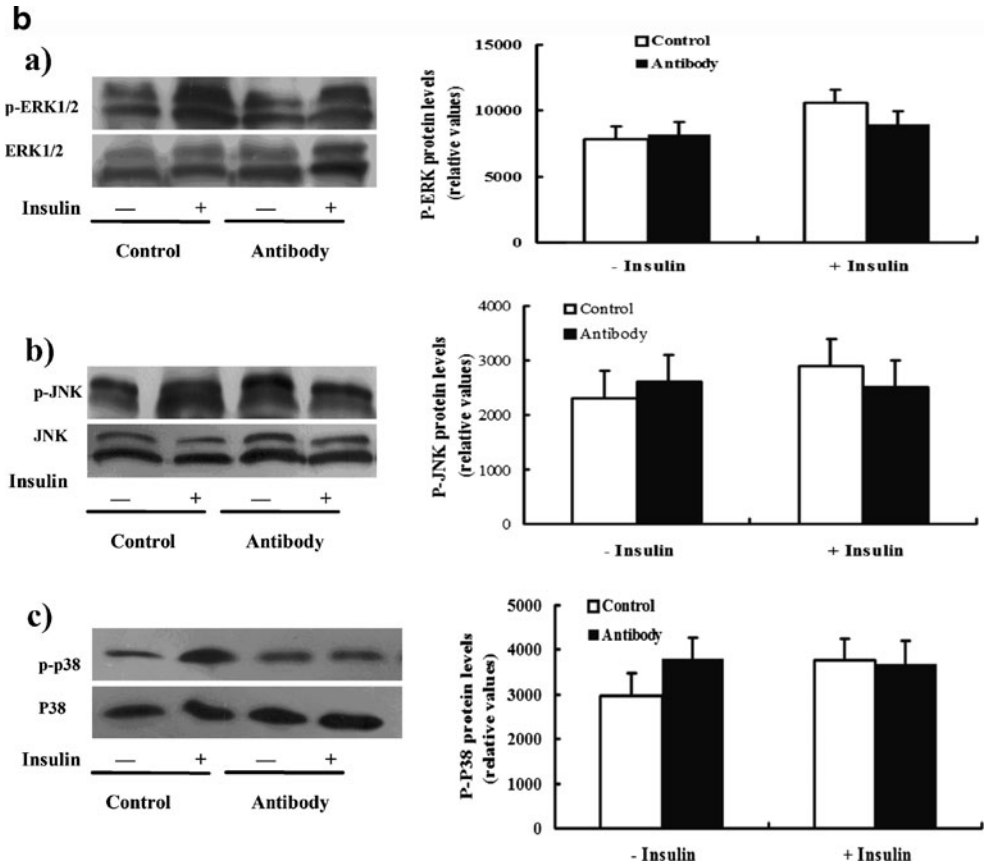

Fig. 2 (continued)

Results

Effects of anti-STEAP4 antibodies on baseline and insulin-stimulated GLUT4 translocation

The cells were used for experiments after 85% had differentiated to the point of having morphological properties of adipocytes. Therefore, following cell differentiation into mature adipocytes, we used the western blot to examine STEAP4 expression (Fig. 1a, Table 1) and the effects of anti-STEAP4 antibodies on insulin-induced GLUT4 translocation to the PM. The results showed that after anti-STEAP4 antibody exposure, insulin-stimulated GLUT4 translocation to the PM decreased without altering baseline GLUT4 translocation or total GLUT4 protein content (Fig. 1b, Table 2).

Effects of anti-STEAP4 antibodies on protein expression and insulin-stimulated phosphorylation of insulin signaling molecules

We next investigated the effect of anti-STEAP4 antibodies on the molecules involved in insulin signaling for glucose uptake. Anti-STEAP4 antibodies resulted in significant inhibition of insulin-induced tyrosine phosphorylation of IRS-1, serine phosphorylation of Akt, and serine phosphorylation of PI3K, whereas anti-STEAP4 antibodies did not affect tyrosine phosphorylation of IR (Fig. 2a). We also evaluated the phosphorylation of signaling molecules downstream from the Ras/MAPK insulin signal pathway: ERK1/2, p38, and JNK (Fig. 2b). We found that anti-STEAP4 antibodies have no effect on insulin-induced phosphorylation of ERK1/2, JNK, and p38. As shown in Fig. 2a and b, no significant differences are present in the total protein content of these signaling molecules.

Effects of STEAP4 antibody on mitochondria morphology, ROS levels, cellular ATP production, and membrane potential ($\Delta\Psi$)

To determine whether mitochondria play a role in adipocyte insulin resistance, we assessed mitochondria ultrastructure in adipocytes following anti-STEAP4 antibody treatment. Notably, cells incubated with antibody displayed condensed mitochondria with twisted and condensed cristae that were smaller compared to those

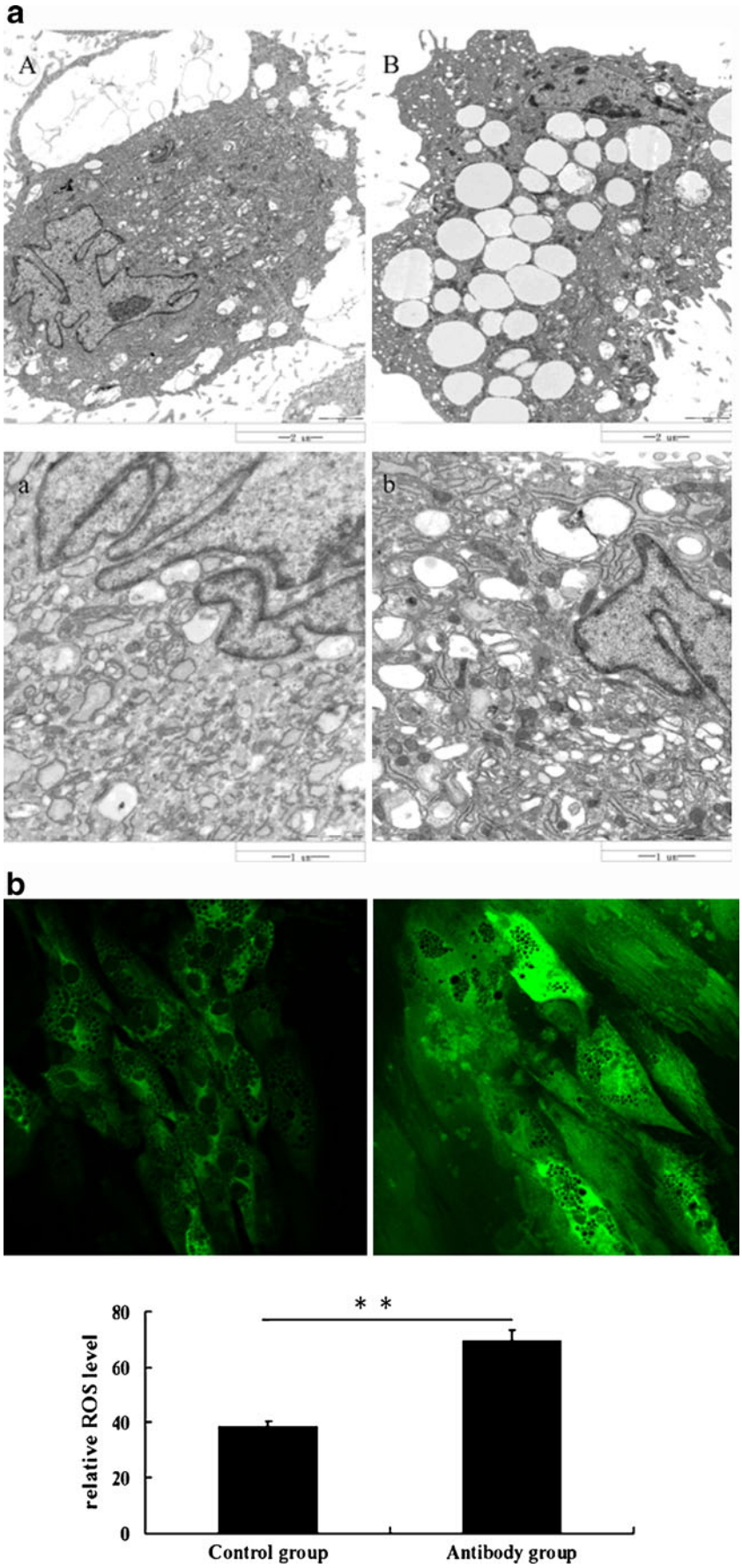

Fig. 3 **a** Effects of STEAP4 antibody on mitochondria morphology. **b** Effects of STEAP4 antibody on ROS levels. **c** Effects of STEAP4 antibody on cellular ATP production. **d** Effects of STEAP4 antibody on membrane potential ($\Delta\Psi$)

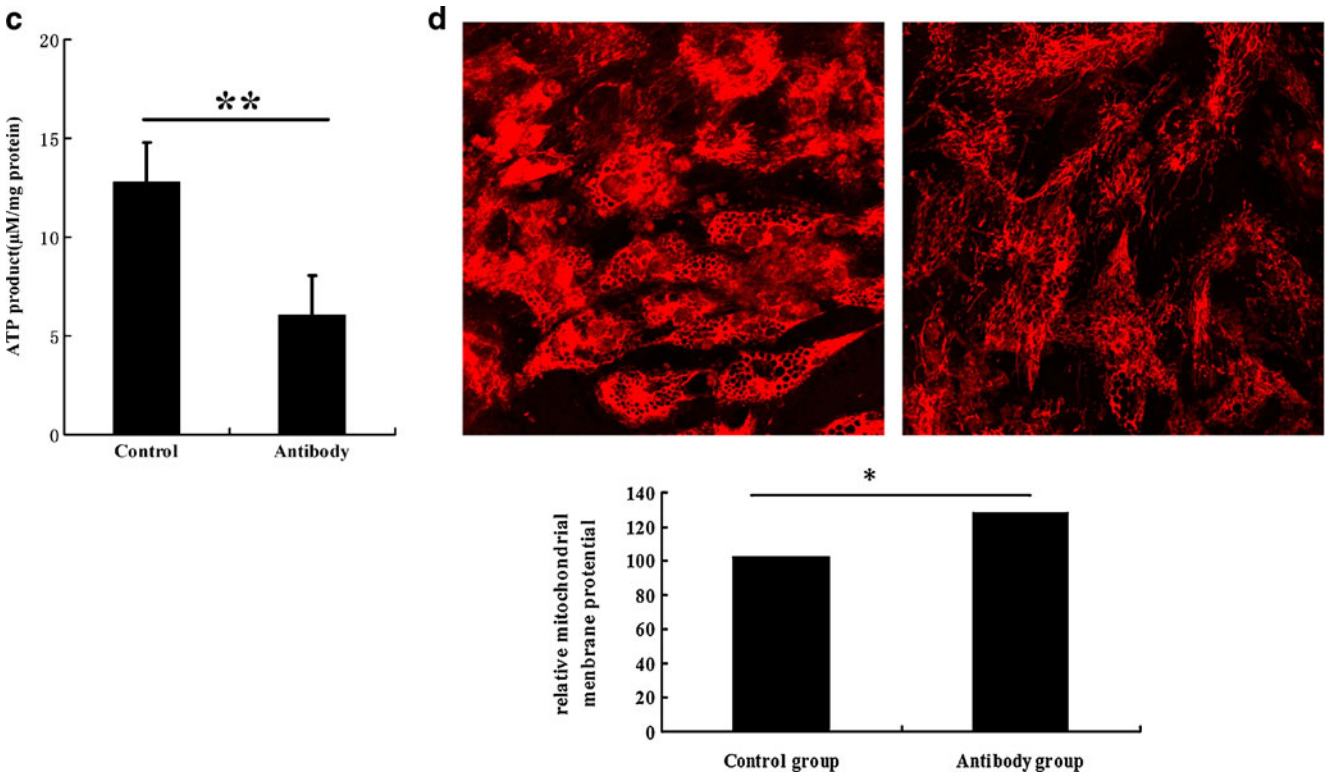

Fig. 3 (continued)

of control adipocytes (Fig. 3a). To elucidate ROS involvement in mitochondria dysfunction within antibody action groups, we probed for the ROS quantities in the 2 groups. As shown in Fig. 3b and Table 3, the STEAP4 antibody dramatically increased ROS levels. Impaired mitochondria may also cause cells to lack sufficient ATP. As expected with abnormal mitochondria, we found total cellular ATP was dramatically decreased in the intervention of anti-STEAP4 antibodies group (Fig. 3c, Table 4). $\Delta\Psi$ is another important indicator of mitochondrial function. Providing additional evidence of mitochondrial dysfunction in the intervention of STEAP4 antibody adipocytes, we determined the adipocyte $\Delta\Psi$ by staining with MitoTracker Red, a mitochondria-selective probe. As shown in Fig. 3d (Table 5), antibody-exposed adipocytes weakly stained with MitoTracker compared to control group cells.

Discussion

Insulin resistance is a pathological state in which insulin activity is impaired in target tissues, including the liver, skeletal muscle, and adipose tissue (Joe Chiba 2009). STEAP4, a novel obesity-related gene in human adipose tissue, is associated with adiposity and insulin resistance (Andjelković et al. 1996; Andjelković et al. 1997; Khayat et al. 2000). In adipocytes, insulin has a multidisciplinary role in how it affects glucose metabolism. However, a primary purpose of insulin is the ability to increase the cellular glucose transport rate (Okada et al. 1994). While anti-STEAP4 antibodies at 0.002 mg/mL could increase reactive oxygen species and reduce mitochondrial membrane potential in human preadipocytes (Knutson 2007), here we found that anti-STEAP4 antibody at this concentration significantly

Table 3 ROS levels ($\bar{x}\pm s$)

groups	Number of samples	Fluorescence intensity
Control	18	38.8837±3.73890
Antibody	18	61.3310±7.01922

Table 4 ATP levels ($\bar{x}\pm s$)

groups	Number of samples	ATP product (μM/mg protein)
Control	18	11.8143±1.34589
Antibody	18	5.0333±1.57739

Table 5 mitochondrial membrane potential levels($\bar{x}\pm s$)

groups	Number of samples	Fluorescence intensity
Control	18	100.2865±1.52826
Antibody	18	129.3813±4.87409

decreases insulin-stimulated glucose transport in mature adipocytes and has no effect on baseline glucose uptake. Insulin-stimulated glucose uptake in adipose tissue is mediated by translocation of insulin-sensitive GLUT4 from intracellular vesicles to the PM. We studied GLUT4 translocation to determine the mechanism by which anti-STEAP4 antibodies decrease insulin-stimulated glucose uptake. The results indicate that STEAP4 affects insulin-stimulated glucose uptake by decreasing GLUT4 translocation to the PM.

The activation of PI3K regulates the dynamic actin rearrangement required for GLUT4 translocation (Korkmaz et al. 2005; Lastra et al. 2008; Martin et al. 1996; Maxwell et al. 1999). A well-documented example of a PI3K downstream target is the protein Ser/Thr kinase, Akt, which is activated via membrane localization (Moldes et al. 2001) and Ser/Thr phosphorylation (Morino et al. 2006; Ohgami et al. 2006). To further investigate the mechanisms by which anti-STEAP4 antibodies decrease both insulin-stimulated GLUT4 translocation and glucose uptake (Ducluzeau et al. 2002), we examined protein content and phosphorylation of the molecules involved in insulin signaling for glucose transport. Insulin receptor-mediated tyrosine phosphorylation of IRS, including IRS proteins and Shc (in the adipose tissue), plays an important role in insulin signaling pathways. Subsequently, we examined the protein content and phosphorylation level of IR, IRS-1, and the MAPKs (ERK, p38, and JNK), located upstream from Akt and playing a central role in metabolic effects of insulin on adipocytes (Ohgami et al. 2006). In this study, we found that anti-STEAP4 antibodies attenuated the degree of phosphorylation of Akt with no effects on the phosphorylation levels of ERK, p38, and JNK. Therefore, we deduced that anti-STEAP4 antibodies affect insulin sensitivity by attenuating Akt phosphorylation. Intriguingly, anti-STEAP4 antibodies caused a decrease to insulin-stimulated tyrosine phosphorylation of IRS-1 without altering tyrosine phosphorylation of IR.

Based on these results, we speculated that the downregulated Akt phosphorylation that results from attenuated tyrosine phosphorylation of IRS-1 may impair insulin-stimulated GLUT4 translocation in STEAP4 antibody action groups. STEAP4 has an N-terminal domain associated with nicotinamide-adenine dinucleotide phosphate (NADP) oxidoreductase coenzyme activity (Andjelković et al. 1996), which provides a potential clue to STEAP4-associated bio-chemical functions. In this study we found that intracellular ROS clearly increased, while the mitochondrial membrane potential and ATP significantly decreased. The increase in ROS may stem from several sources, and the NAD(P)H oxidase enzyme is capable of significant ROS production. This prompted us to propose that mitochondrial dysfunction caused by increased ROS may have a role in insulin resistance (Kim et al. 2008). In addition, the metabolic actions of insulin resistance may be mediated by IRS-1 degradation via redox-sensitive serine phosphorylation of IRS-1 and decreased downstream signal transduction (Okada et al. 1994; Park et al. 2006). Guido Lastra et al., also found that ROS is apparently associated with reduced IRS-1 levels, reduced Tyr phosphorylation of IRS-1 and Akt phosphorylation/activation, and insulin-stimulated glucose transport (Sears et al. 2009). Additionally, previous studies also suggested an NADPH-dependent H_2O_2-generating system was associated with human and rodent adipocyte plasma membranes (Weiner and Carter 2005; Wellen et al. 2007) and the reactive oxygen species (ROS) played a vital role in obesity and in obesity-related insulin resistance (Zhang et al. 2008). Taken together, our results suggested that STEAP4 might be involved in ROS-related mitochondrial dysfunction and the reduced level of IRS-1 may eventually contribute to the development of obesity and obesity related IR.

In conclusion, our data provide evidence that: (i) in response to insulin, STEAP4 regulates the function of IRS-1, PI3K, and Akt and decreases GLUT4 translocation and glucose uptake; (ii) ROS-related mitochondrial dysfunction may be due to the reduction of IRS-1 correlation with PI3K signaling pathway, leading to insulin resistance. These observations highlight the potential role of STEAP4 in glucose homeostasis and possibly the pathophysiology of type 2 diabetes related to obesity and may provide new insights into the mechanisms of insulin resistance in obesity.

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