Effect of bisphenol A on SOCS-3 and insulin signaling transduction in 3T3-L1 adipocytes

YUE-E DAI, WEI CHEN, HUMIN QI and QIAN-QI LIU

Department of Endocrinology, Nanjing Children's Hospital, Affiliated With Nanjing Medical University, Nanjing, Jiangsu 210008, P.R. China

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Abstract. The aim of the present study was to investigate whether environmental endocrine disrupting chemical, bisphenol A (BPA), affects secretion of suppressor of cytokine signaling 3 (SOCS-3) and insulin signaling transduction in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated for 0, 2, 6, 12 and 24 h with BPA at 80 μM in serum-deprived medium. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to detect the mRNA expression levels of SOCS-3 and protein expression levels of SOCS-3, insulin receptor substrate 1 (IRS-1), phosphorylated (p)-IRS-1, Akt and p-Akt. The levels of p-IRS-1, Akt and p-Akt in cultures treated for 6 h with BPA were also analyzed by immunofluorescence. The SOCS-3 mRNA and protein expression levels were decreased in the 6, 12 and 24 h groups. The levels of p-IRS-1 and p-Akt protein were markedly downregulated, while the level of IRS-1 and Akt protein remained unaltered among these groups, which was consistent with the results observed using immunofluorescence. BPA may inhibit insulin signal transduction and result in the occurrence of insulin resistance via promoting the expression of SOCS-3.

Introduction

The incidence of diabetes has notably increased in recent years, and it is estimated that ~380 million patients will be diagnosed worldwide by 2025 (1). Insulin resistance (IR), a key feature of type 2 diabetes, is a state in which insulin has a reduced ability to mediate glucose homeostasis in its major target tissues, resulting in compensatory hyperinsulinemia. Previous studies have indicated that various signaling pathways, particularly insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K)/Akt, are key in mediating the occurrence of IR (2,3).

Correspondence to: Dr Qian-Qi Liu, Department of Endocrinology, Nanjing Children's Hospital, Affiliated With Nanjing Medical University, 72 Guangzhou Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: 18951769617@163.com

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Insulin action involves a series of signaling transduction pathways, initiated by insulin binding to its receptor, which initiates receptor autophosphorylation and activation of the receptor tyrosine kinase, resulting in tyrosine phosphorylation of IRS-1, a substrate of the insulin receptor (4,5). Subsequently, phosphorylation of IRS-1 leads to activation of PI3K and Akt and its downstream signaling molecules, all of which are important in the promotion of the synthesis of glycogen and regulation of glucose homeostasis (6). Thus, a state of IR often suggests the attenuation or failure of insulin signaling transduction by inhibiting the phosphorylation of key molecules.

Bisphenol A (BPA) is widely used as a plasticizer and stabilizer in the manufacture of consumer products, however, it is considered an endocrine disrupting chemical (EDC). Exposure to EDCs is proposed to be involved in the etiology of IR and associated metabolic disorders (7). Data from the U.S. National Health and Nutritional Examination Survey 2003-2008 reported a positive association between urinary BPA levels and an increased prevalence of diabetes mellitus independent of traditional diabetes risk factors (8), consistent with research findings in China (9). In addition, previous studies have elucidated the mechanisms by which BPA provoked IR by affecting glucose transport (10,11), adiponectin secretion (12), adipocyte differentiation and lipid accumulation (13).

Furthermore, suppressor of cytokine signaling 3 (SOCS-3), a negative regulator of insulin signaling, is known to be associated with IR. A previous study demonstrated resistin induced IR in HepG2 cells via induction of SOCS-3 expression (14). It has also been previously reported that the expression levels of SOCS-3 were markedly increased in mice with IR induced by a high fat diet (15), which may contribute to increased serine phosphorylation of IRS-1. Insulin signaling transduction is impaired by inhibiting the activation of tyrosine phosphorylation of IRS-1 (16). However, to the best of our knowledge, no investigation into the effect of BPA on SOCS-3 has been conducted. The aim of the current study was to investigate whether BPA modulates SOCS-3 production and affects insulin signaling transduction in 3T3-L1 adipocytes.

Materials and methods

Reagents and antibodies. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

BPA, dexamethasone, insulin and 3-isobuty-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-SOCS-3 antibody (cat. no. 2923), rabbit monoclonal anti-IRS-1 antibody (cat. no. 2390), rabbit polyclonal anti-Akt antibody (cat. no. 9272) and rabbit polyclonal anti-phosphorylated (p)-Akt (Ser473) antibody (cat. no. 9271) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; dilution, 1:1,000 for western blotting). Goat polyclonal anti-p-IRS-1 (Tyr 632) antibody (cat. no. sc-17196) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; dilution, 1:200). Mouse monoclonal antibodies against β-actin (cat. no. ab8226; dilution, 1:1000), horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (cat. no. ab6721; dilution 1:200), horseradish peroxidase goat anti-mouse IgG (cat. no. ab6789; dilution, 1:200), horseradish peroxidase-conjugated donkey anti-goat IgG (cat. no. ab6885; dilution, 1:100), fluorescein isothiocyanate (FITC)-conjugated polyclonal donkey anti-goat IgG (cat. no. ab6881; dilution, 1:200), FITC-conjugated polyclonal goat anti-rabbit IgG (cat. no. ab6717; dilution, 1:100) and cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (cat. no. 97075; dilution, 1:100) secondary antibodies were purchased from Abcam (Cambridge, MA, USA).

Cell culture and treatment. The 3T3-L1 mouse preadipocyte cell line was obtained from the American Type Culture Collection (Manassas, VA, USA), and the cells were cultured and differentiated into adipocytes as described previously (17). Briefly, the cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin (Beyotime Institute of Biotechnology, Haimen, China) and 100 mg/ml streptomycin (Beyotime Institute of Biotechnology) at 37˚C in a humidified atmosphere of 5% CO_2. The cells were exposed to standard differentiation inducers 48 h after confluency was reached. The inducer used was DMEM containing 0.5 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin for 48 h (from day 0 to 2). The medium was then changed and supplemented with 10 μg/ml insulin only for the following 48 h (from day 2 to 4). Thereafter, the medium was replaced by growth medium and changed every 2 days. At 10 days after the induction of differentiation, >80% of cells exhibited typical morphology and biochemical properties of adipocytes. Following overnight incubation in serum-free DMEM, 3T3-L1 adipocytes were treated with 80 μM BPA diluted in DMEM for 0, 2, 6, 12 and 24 h respectively.

RNA preparation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from 3T3-L1 adipocytes treated with BPA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols and quantified spectrophotometrically by measuring absorbance at wavelengths 260 and 280 nm on a BioPhotometer spectrophotometer (Eppendorf, Hamburg, Germany). RT was conducted using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland) from 1 μg RNA as described by the manufacturer's protocols. The temperature protocol for the reaction was 25˚C for 10 min, 55˚C for 30 min and 85˚C for 5 min. qPCR was performed to determine the relative mRNA expression levels of SOCS-3. β-actin served as an internal control for normalization. Specific mRNAs were amplified in FastStart Universal SYBR Green Master mix (Roche Diagnostics) using the ABI Prism 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) sequence detector with the following thermocycling conditions: 50˚C for 2 min; 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The primers (Invitrogen; Thermo Fisher Scientific, Inc.) were as follows: Forward, 5'-ATGGTCACCCACAGCAAGTTT-3' and reverse, 5'-TCCAGTAGAATCCGCTCTCCT-3' for SOCS-3; and forward, 5'-CTACAATGAGCTGCGTGTGG-3' and reverse, 5'-AAGGAAGGCTGGAAGAGTGC-3' for β-actin. Three data points were used and the experiment was replicated three times and the data was analyzed using the $2^{-\Delta\Delta Cq}$ method (18).

Protein extraction and western blot analysis. At 0, 2, 6, 12 and 24 h, the cells treated with BPA were washed twice with ice-cold phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.) and lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) supplemented with 1% protease inhibitor (GeneChem Co., Ltd., Shanghai, China) and 1% phosphatase inhibitor (GeneChem Co., Ltd.). Total and phosphorylated proteins were extracted and concentration was determined using the BCA Protein assay kit (Beyotime Institute of Biotechnology). Following 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 30 min at 80 V followed by 120 V, the protein bands (20 μg/lane) were electrophoretically transferred onto polyvinyl difluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). The blots were blocked for 2 h at room temperature in Tris-buffered saline (Beyotime Institute of Biotechnology), with 0.1% Tween 20 (Sigma-Aldrich; TBS-T) and 5% non-fat dried milk and subsequently incubated overnight at 4˚C with the primary antibodies against SOCS3, IRS-1, p-IRS-1, Akt, p-Akt and β-actin. Following washing in TBS-T buffer, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Following washing in TBS-T, antigen-antibody complexes were detected using Amersham ECL Western Blotting Detection reagent (GE Healthcare Life Sciences) and visualized with the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified using the Quantity One image software (version 4.31; Bio-Rad Laboratories, Inc.).

Immunocytochemistry. After 6 h treatment with BPA, 3T3-L1 adipocytes were fixed for 30 min with 4% paraformaldehyde (Sigma-Aldrich) at room temperature, and then rinsed three times for 5 min with PBS. The cells were permeabilized for 10 min in 0.1% Triton X-100 (Sigma-Aldrich), then again rinsed twice for 5 min in PBS, and blocked for 1 h in PBS with 5% bovine serum albumin (Sigma-Aldrich) at room temperature. Antibodies against p-IRS-1 (dilution, 1:200), Akt (dilution, 1:200) and anti-p-Akt (dilution, 1:200) were incubated with the cells at 4˚C overnight. The cells were then incubated with FITC-conjugated goat anti-rabbit IgG (1:500) or Cy3-conjugated goat anti-rabbit IgG (1:500) at room temperature for 2 h, followed by washing in PBS. The cells were stained with DAPI (Sigma-Aldrich) for 3 min and images were captured using a Nikon Eclipse Ti-S fluorescent inverted microscope (Nikon Corporation, Tokyo, Japan) at magnification x200.

Statistical analysis. All data are expressed as the mean ± standard deviation. Differences among 3-5 independent groups

were statistically evaluated using one-way ANOVA, while significant differences between 2 independent groups were analyzed using Student's t-test. Statistical analysis was conducted using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

BPA increases SOCS-3 expression levels in 3T3-L1 adipocytes. The mRNA expression levels of SOCS-3 were analyzed using RT-qPCR and the results indicated that BPA treatment at 80 μM significantly increased mRNA expression levels in time-dependent manners (P<0.01 at 2-12 h and P<0.05 at 24 h; Fig. 1). In order to further investigate the protein expression of SOCS-3, western blotting was performed and the result is presented in Fig. 2. RT-qPCR and western blot analysis indicated that BPA significantly increased SOCS-3 mRNA and protein expression levels after 6 and 12 h of treatment compared to 0 h (P<0.01). In addition, the expression levels of SOCS-3 mRNA and protein were overexpressed after 24 h of treatment with BPA (P<0.05).

BPA alters protein expression levels of insulin signaling molecules in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with 80 μM BPA for 0, 2, 6, 12 and 24 h and the expression levels of IRS-1, p-IRS-1, Akt and p-Akt were analyzed by western blotting. As presented in Fig. 3, there were no significant differences in the protein expression levels of IRS-1 among these groups. However, BPA decreased the expression levels of p-IRS-1 at 6 h of treatment compared with 0 h (P<0.01). Similar effects were also observed in the level of Akt (Fig. 4). However, a significant decrease in expression levels of p-Akt was observed following treatment with BPA for 2 to 24 h (P<0.01; Fig. 4). These results suggest that BPA markedly decreased the expression levels of insulin signaling molecules in 3T3-L1 adipocytes.

BPA decreased expression levels of p-IRS-1 and p-Akt. To further elucidate the effect of BPA, immunocytochemistry was conducted to investigate the expression levels of the insulin signaling molecules. Consistent with the results from the western blotting, p-IRS-1 (Fig. 5) and p-Akt (Fig. 6) expression levels were markedly decreased in BPA-treated cells compared with control cells, while expression levels of Akt did not exhibit a marked change (Fig. 6).

Discussion

Adipose tissue is important in insulin sensitivity and basal metabolic rate. Thus, 3T3-L1 adipocytes were selected to investigate the effects of BPA on SOCS-3 and insulin signaling transduction. In the current study, it was observed that BPA significantly increased SOCS-3 secretion in 3T3-L1 adipocytes (P<0.01) and decreased the expression of key molecules involved in the IRS-1/PI3K/Akt signaling pathway.

BPA, in addition to other environmental estrogens, has become a public health concern due to deleterious effects on energy balance and glucose homeostasis (19). The present study indicates that BPA exposure impairs insulin signaling in peripheral tissues and may be a risk factor for the development of type 2 diabetes (20). During the early stages of life, BPA exposure may impair pancreatic development and result in adults susceptible to diabetes (21). In epidemiological studies in humans, >93% of US adults have detectable BPA levels in urine, higher levels are particularly observed in the population with diabetes, hypertension and obesity (22). In animal models, BPA exposure in pregnant rats increased their offspring's body weight, and the levels of fasting blood glucose and serum insulin, which may predispose them to IR (23). Data from a previous study demonstrated that BPA exhibited estrogen-like activities via binding to estrogen receptors (ERs), non-classical membrane ERs, G-protein-coupled receptor 30 and estrogen-related receptors (24). There were, however, few studies that had investigated the effect of BPA on insulin signal transduction, thus, the present study aimed to investigate the association between BPA and the IRS-1/PI3K/Akt signaling pathway.

As previously described, IR may be induced by the inhibition of insulin signaling transduction. In the current study, the results of the western blotting indicated that BPA significantly decreased the expression levels of p-IRS-1 and p-Akt (P<0.01), which are key in insulin-stimulated glucose transport (25). The decrease in protein expression levels of p-IRS-1 and p-Akt were further shown by immunocytochemistry. *In vitro*, the cellular uptake of glucose into the cells by glucose transporters requires insulin and receptor-mediated tyrosine phosphorylation of IRS-1 (26), which is key in insulin signal transduction and affects insulin signaling by regulating protein presentation, post-translational modification and subcellular localization of proteins, particularly in phosphorylation/dephosphorylation of post-translational modification (27). IRS-1 is closely associated with PI3K activation, which is responsible for activation of the Akt signaling cascade (28). It is generally accepted that impaired tyrosine phosphorylation of IRS-1 is responsible for reduced insulin signaling and impaired downstream PI3K/Akt signal transduction (29). The downregulated phosphorylation of Akt resulting from attenuated tyrosine phosphorylation of IRS-1 may impair GLUT4 translocation and glucose uptake. A previous study has indicated that insulin-stimulated Akt phosphorylation was suppressed in skeletal muscle and livers of BPA-treated pregnant mice, these mice then suffered from metabolic disorders associated with glucose homeostasis (30). The present study suggests that 80 μM BPA may inhibit the IRS-1/PI3K/Akt signaling pathway, which results in IR.

To further investigate the underlying mechanisms of BPA induced impairment of insulin signaling transduction, the mRNA and protein expression levels of SOCS-3 were investigated by RT-qPCR and western blotting. The results demonstrated that BPA markedly increased SOCS-3 mRNA and protein expression levels in a time-dependent manner. In addition, it was observed that tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt was decreased as demonstrated by a decrease in the expression levels of these proteins following the treatment with BPA. This was consistent with the increased expression levels of SOCS-3 at the same time points.

SOCS-3 is one member of the SOCS protein family, which is overexpressed in insulin-sensitive tissues from patients

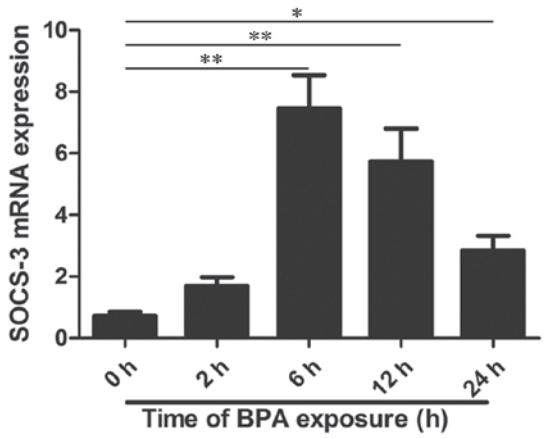

Figure 1. mRNA expression levels of SOCS-3. The mRNA expression levels of SOCS-3 were detected by reverse transcription-quantitative polymerase chain reaction at 0, 2, 6, 12 and 24 h after BPA treatment. *P<0.05, **P<0.01. BPA, bisphenol A; SOCS-3, suppressor of cytokine signaling 3.

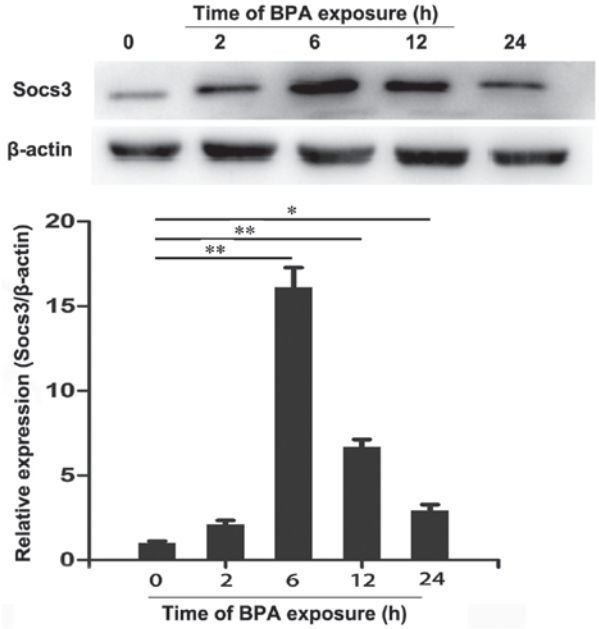

Figure 2. Western blot analysis of SOCS-3 expression. The expression levels of SOCS-3 and β-actin were detected by western blot at 0, 2, 6, 12 and 24 h after BPA treatment. *P<0.05, **P<0.01. BPA, bisphenol A; SOCS-3, suppressor of cytokine signaling 3.

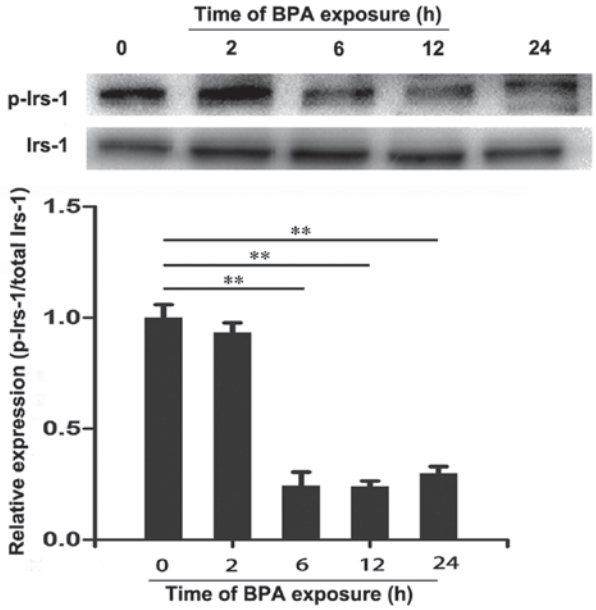

Figure 3. Western blot analysis of p-IRS-1 and IRS-1. The expression levels of p-IRS-1 and IRS-1 were detected by western blotting at 0, 2, 6, 12 and 24 h after BPA treatment. **P<0.01. BPA, bisphenol A; IRS-1, insulin receptor substrate 1; p, phosphorylated.

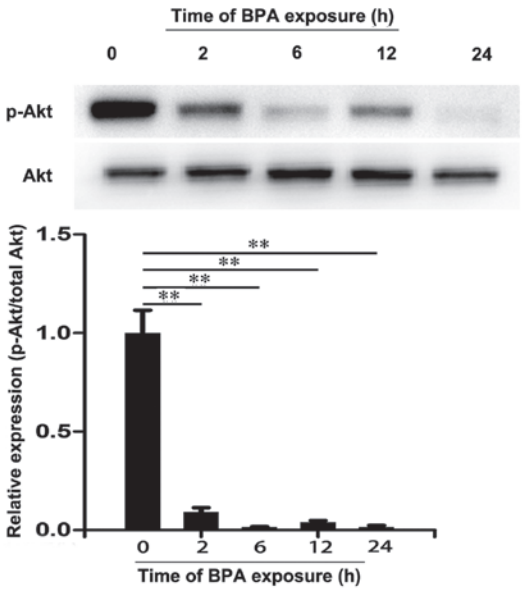

Figure 4. Western blot analysis of p-Akt and Akt. The expressions of p-AKT and AKT were detected by western blotting at 0, 2, 6, 12 and 24 h after BPA treatment. **P<0.01. BPA, bisphenol A; p, phosphorylated.

with type 2 diabetes and IR and animal models of the conditions (31,32). Previous studies have demonstrated SOCS-3 binds via the SH2 domain to tyrosine phosphorylation sites on cytokine receptors to inhibit inflammatory signal transduction (33). In the skeletal muscle of obese Zucker rats, SOCS-3 protein concentration and co-localization of SOCS-3 with IRS-1 is notably increased, while tyrosine phosphorylation of IRS-1 was decreased and serine phosphorylation of IRS-1 was increased (34). Furthermore, mice with muscle-specific deletion of SOCS-3 were protected against the development of hyperinsulinemia and IR due to enhanced skeletal muscle IRS-1 and Akt phosphorylation (16). Similarly, genetic deletion of SOCS-3 from mouse liver also results in enhanced insulin signaling due to increased IRS-1 phosphorylation (35). These studies suggest SOCS-3 interferes with insulin signaling and results in IR by inhibiting tyrosine phosphorylation of IRS-1.

In conclusion, BPA significantly increases mRNA and protein expression levels of SOCS-3 and decreases the phosphorylation of IRS-1 and Akt. Based on these results, the present study hypothesizes that BPA may inhibit insulin signal transmission and lead to the development of IR via promoting the expression of SOCS-3 and preventing tyrosine phosphorylation of IRS-1. The present study provides a novel insight into the mechanism by which BPA induces IR.

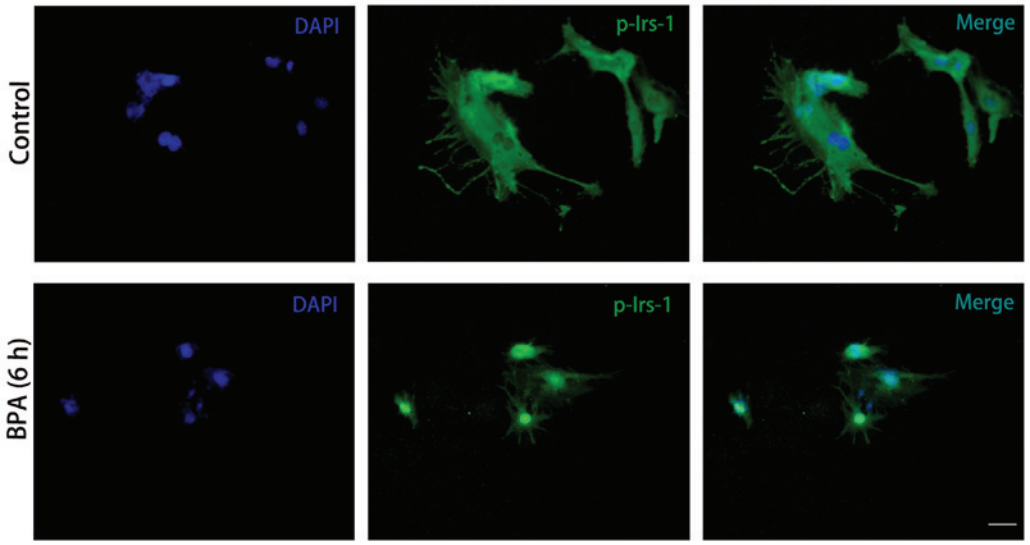

Figure 5. Immunocytochemistry of p-IRS-1. Expression levels of p-IRS-1 were markedly lower in BPA-treated cells after 6 h. Bar =50 μm. Magnification, x200. BPA, bisphenol A; IRS-1, insulin receptor substrate 1.

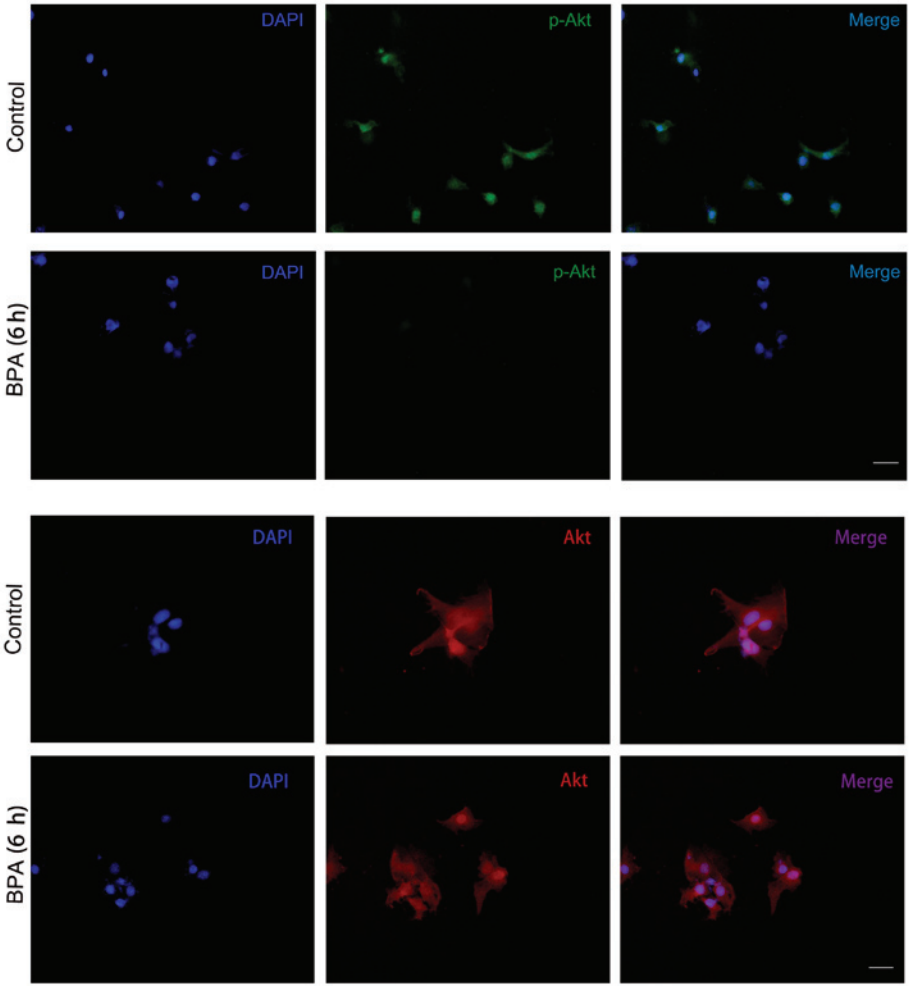

Figure 6. Immunocytochemistry of p-Akt and Akt. Expression levels of p-Akt were markedly lower in BPA-treated cells after 6 h, while Akt expression exhibited no marked change. Bar =50 μm. Magnification, x200. BPA, bisphenol A; p, phosphorylated.

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