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Effect of Bisphenol A on Insulin-Mediated Glucose Metabolism *In Vivo* and *In Vitro*

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Accepted 1 November 2008

Abstract

Bisphenol A (BPA), an environmental endocrine disrupter, enters the human body continuously in food and drink. Young children are likely to be more vulnerable than adults to chemical exposure due to the immaturities of their organ systems, rapid physical development, and higher ventilation, metabolic rates, and activity levels. The direct effect of BPA on peripheral tissue might also be of importance to the development of insulin resistance. However, the influence that BPA has on insulin signaling molecules in skeletal muscle has not been previously investigated. In this study, we examined the effect of BPA on fasting blood glucose (FBG) in post-weaned Wistar rats and on insulin signaling proteins in C2C12 skeletal muscle cells. Subsequently, we investigated the effects of BPA on insulin-mediated Akt phosphorylation in C2C12 myotubes. In rats, BPA treatment (0.1-1,000 ng/mL for 24 hours) resulted in the increase of FBG and plasma insulin levels, and reduced insulin-mediated Akt phosphorylation. Furthermore, the mRNA expression of insulin receptor (IR) was decreased after 24 hours of BPA treatment in C2C12 cells in a dose-dependent manner, whereas the mRNA levels of other insulin signaling proteins, including insulin receptor substrate-1 (IRS-1) and 5′-AMP-dependent protein kinase (AMPK), were unaffected. Treatment with BPA increased GLUT4 expression and protein tyrosine phosphatase 1B (PTP1B) activity in C2C12 myotubes, but not in protein levels. We conclude that exposure to BPA can induce insulin resistance by decreasing IR gene expression, which is followed by a decrease in insulin-mediated Akt activation and increased PTP1B activity.

Keywords: Bisphenol A, Insulin resistance, Child, Akt, IR, PTP-1B

Bisphenol A (2,2-bis-(4-hydroxyphenyl)propane; BPA) is present ubiquitously in the environment, and continues to be used as a basic ingredient in the manufacture of polycarbonate and epoxy resin[1,2]. Moreover, it has been reported that exposure to extremely low doses of endocrine disrupting chemicals during fetal and early postnatal development can affect endocrine systems[3]. Furthermore, the direct effect of BPA on peripheral tissue might also be of importance in terms of the development of insulin resistance. Type 2 DM is increasing in the pediatric population, although the primary defect associated with the development of type 2 DM is unknown, impairments in insulin action in skeletal muscle is known to contribute to the pathogenesis of type 2 DM. Insulin stimulates glucose transporter (GLUT4) translocation to the plasma membrane, glucose uptake, and glucose incorporation into glycogen by activating a cascade of signaling events[4-6]. Insulin mediates its cellular responses in target tissues (liver, muscle and adipose) by binding to insulin receptor (IR), a member of the receptor tyrosine kinase superfamily[7]. Upon binding to its receptor, insulin causes rapid autophosphorylation and activation of the receptor's intrinsic tyrosine kinase activity. The stimulation of Akt phosphorylation and activity is a hallmark of the action of insulin in skeletal muscle[8]. Activation of Akt is required and may be sufficient for the stimulation of glucose transport and glycogen metabolism, gene expression, cell survival, proliferation, and protection from apoptosis. Akt is also required for the insulin-induced translocation of GLUT4 to the plasma membrane. In skeletal muscle, glucose transport can be activated by at least two separate

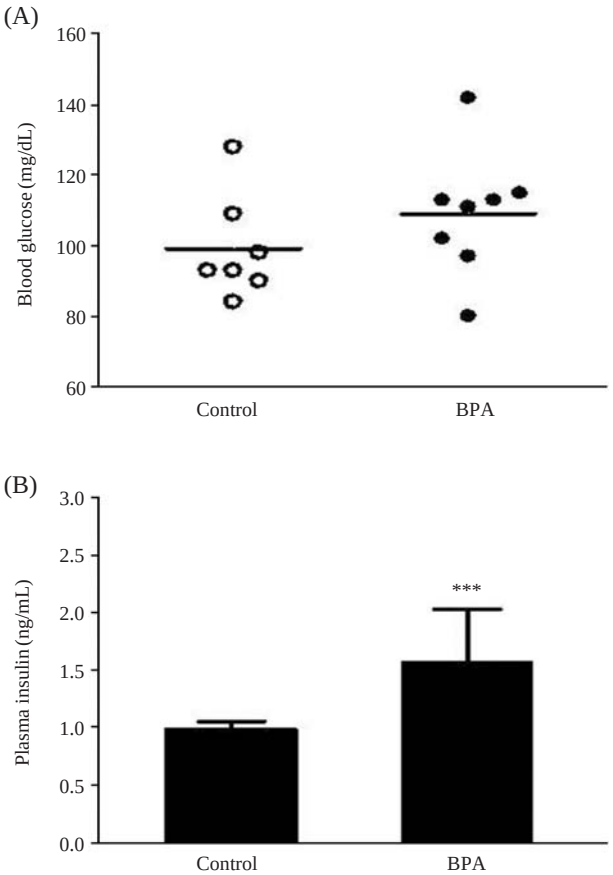

Figure 1. Effect of BPA on fasting blood glucose and plasma insulin levels. Rats were administered BPA using osmotic minipumps (Alzet models 1007D) implanted to deliver BPA (0.5 μg/μL/h) continuously for 7 days. (A) Blood glucose and (B) plasma insulin levels were determined after an overnight (12 h) fast. ***$P < 0.0001$ compared with the control group.

pathways, one stimulated by insulin, insulin mimicking agents, and insulin-like growth factors, and one activated by muscle contraction/exercise and hypoxia[9]. The activation of 5′-AMP-dependent protein kinase (AMPK) has been found to regulate insulin-independent glucose transport elicited in response to metabolic stress[10]. AMPK also functions as a metabolic switch that phosphorylates key target proteins along diverse metabolic pathways, which include hepatic lipid metabolism, adipocyte lipolysis, and skeletal muscle fatty acid oxidation and glucose transport.

Therefore, in this study, we investigated the *in vivo* effect of BPA on glucose homeostasis in Wistar rats and its *in vitro* effects on several insulin signaling molecules in mouse skeletal muscle C2C12 myotubes.

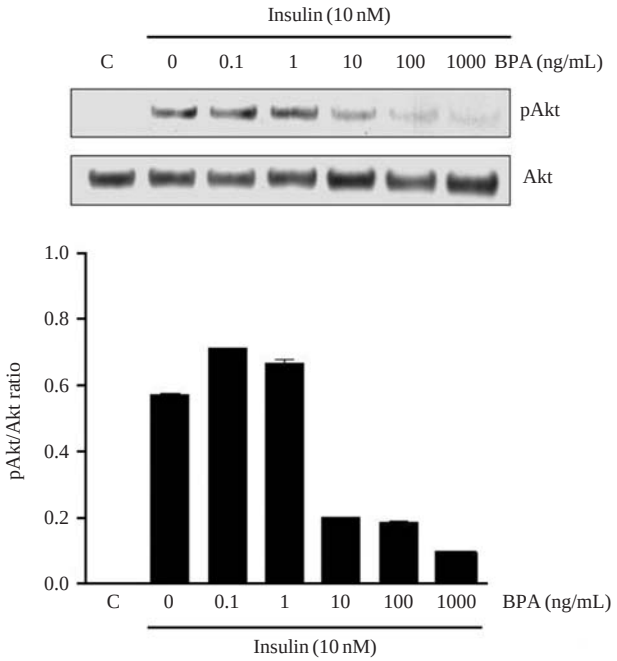

Figure 2. Effect of BPA on Akt phosphorylation. C2C12 cells were pretreated with various concentrations (0.1-1,000 ng/mL) of BPA for 24 hours and then stimulated with 10 nM insulin for 2 minutes. Akt phosphorylation levels were determined by Western blotting using anti-phospho-Akt and anti-Akt antibodies (a representative band is shown). pAkt/Akt ratios were determined using Bio-Profil Bio1D software.

Effect of BPA on Glucose Homeostasis in Wistar Rats

To investigate the effect of BPA on the regulation of glucose homeostasis by insulin *in vivo*, rats were treated with BPA (0.5 μg/μL/h) for 7 days using a subcutaneously implanted Alzet 1007D osmotic pump. Treatment of animals with BPA significantly increased plasma insulin levels ($P < 0.0001$) and marginally increased fasting blood glucose (Figure 1). Moreover, marked inter-individual variations were observed in fasting plasma insulin levels, particularly in BPA-treated animals.

Effect of BPA on Insulin-stimulated Akt Phosphorylation in C2C12 Myotubes

It has been shown that Akt activation is critical for insulin sensitivity in muscle. To investigate the effects of BPA on peripheral insulin resistance, and specifically on insulin sensitivity in muscle, Akt phosphorylation was analyzed in skeletal muscle cells C2C12 myotubes. As was expected, treatment of cells with 10 nM insulin for 2 min caused a marked increase in Akt phosphorylation, whereas pretreatment with BPA (0.1-1,000 ng/mL) for 24 hours caused a significant

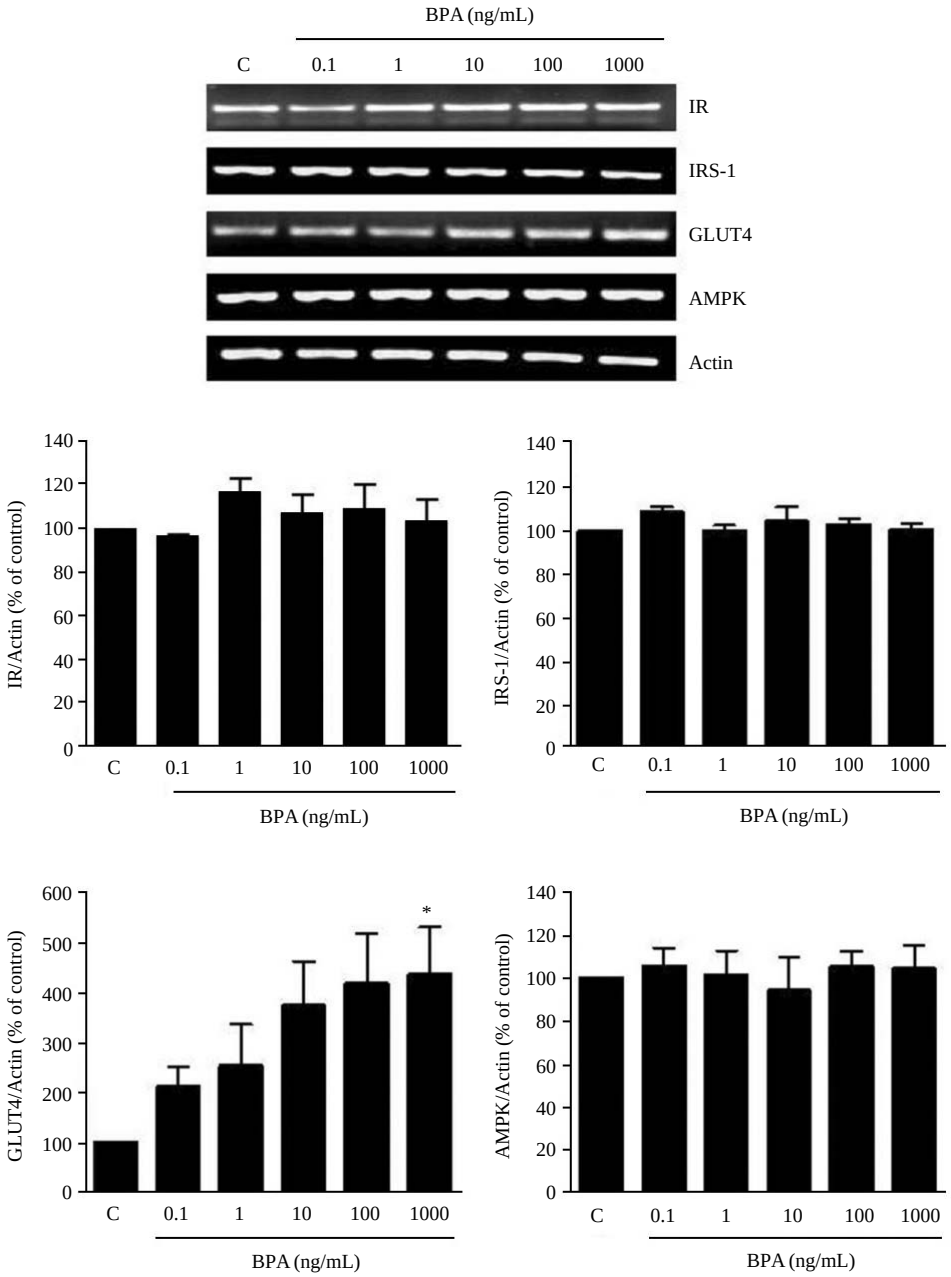

Figure 3. Effect of 12 h of BPA treatment on the mRNA expressions of IR, IRS-1, GLUT4, and AMPK. C2C12 cells were treated with BPA (0.1-1,000 ng/mL) for 12 hours. mRNA expressions were determined by RT-PCR, using actin as a normalization control (a representative band is shown). Relative intensities were evaluated using Bio-Profil Bio1D software. *$P <$ 0.05 compared with the control.

dose-dependent reduction in insulin-mediated Akt phosphorylation (Figure 2).

Effect of BPA on the mRNA Expressions of Insulin Signaling Molecules in C2C12 Myotubes

Next, we determined whether BPA affects the gene expressions of proteins involved in insulin signaling pathways, namely, IR, IRS-1, and GLUT4, in C2C12 myotubes. Treatment of cells with BPA (0.1-1,000 ng/mL for 12 hours) increased GLUT4 expression ($P < 0.05$, Figure 3). As shown in Figure 4, the level of IR mRNA was reduced by BPA for 24 hours in a dose-dependent manner ($P < 0.05$), whereas the mRNA expressions of IRS-1 and AMPK were unaffected.

Effect of BPA on PTP1B Activity and Protein Expression in C2C12 Myotubes

The overactivation of tyrosine phosphatases is another possible mechanism of insulin signal down-regulation. One key phosphatase, protein tyrosine

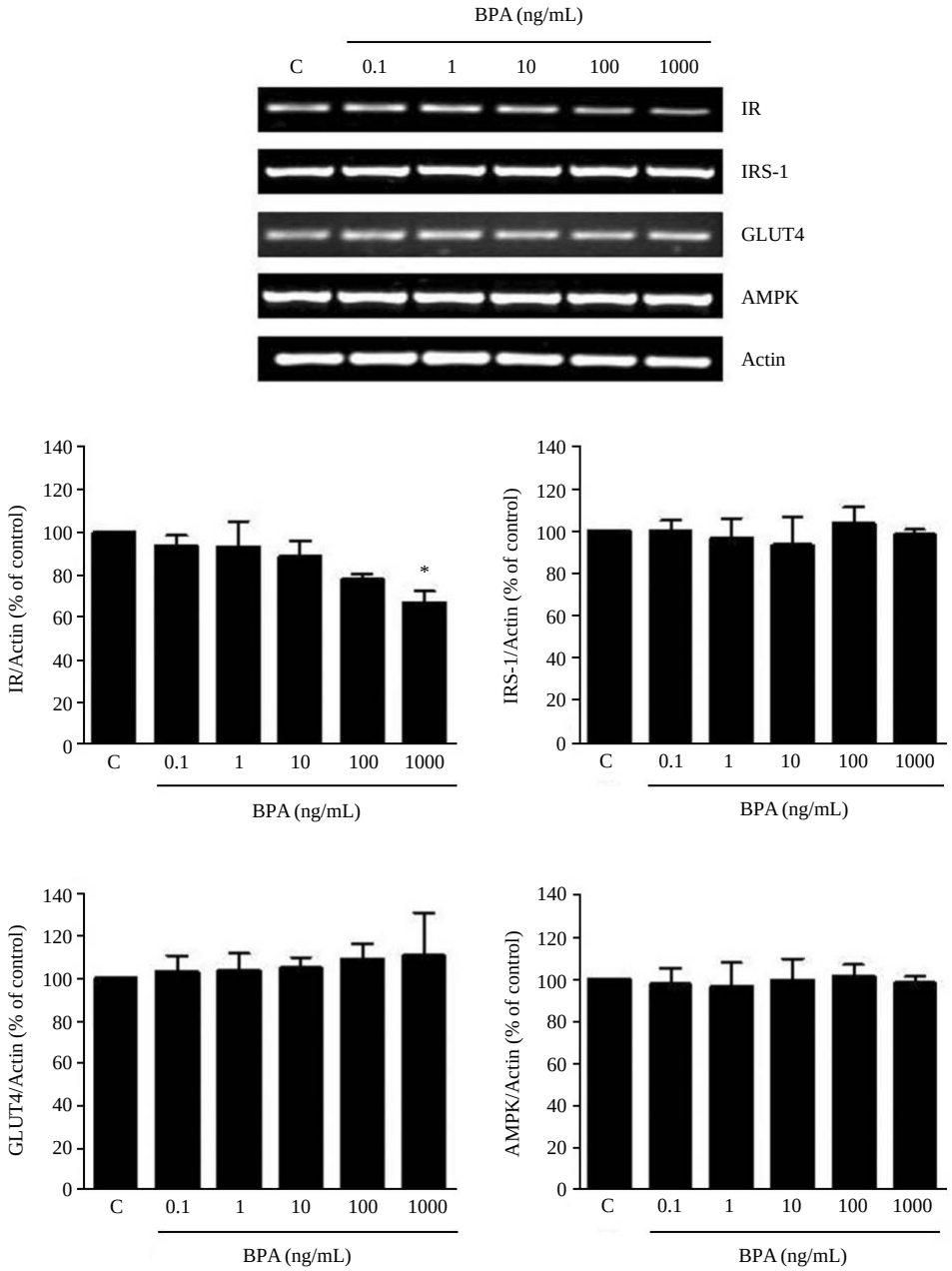

Figure 4. Effect of 24 h of BPA treatment on mRNA expressions of IR, IRS-1, GLUT4, and AMPK. C2C12 cells were treated BPA (0.1-1,000 ng/mL) for 24 hours. mRNA expressions were measured by RT-PCR using actin as a normalization control (a representative band is shown). Relative intensities were evaluated using Bio-Profil Bio1D software. *$P <$ 0.05 compared with the control.

phosphatase 1B (PTP1B), has been implicated in the negatively regulation of insulin receptor (IR) and of its substrates, such as, IRS-1 and -2, which it achieves by dephosphorylating them. Therefore, we tested PTP1B activity in C2C12 myotubes treated with a BPA (100 ng/mL) for various times (0.5, 4, 12, and 24 h). Treatment of cells with BPA for 4 hours significantly increased PTP1B activity, and this increased activity returned to baseline level at 12 hours to 24 hours after treating cells with BPA (Figure 5A). The protein levels of PTP1B were not significantly changed by BPA over 24 hours (Figure 5B).

Discussion

BPA is used to manufacture many food and beverage containers, such as, aluminium and tin cans, and baby bottles. The heating of cans to sterilize food, and the presence of acidic or basic foods or beverages in cans or polycarbonate containers, and the repeated washing of polycarbonate products have all been

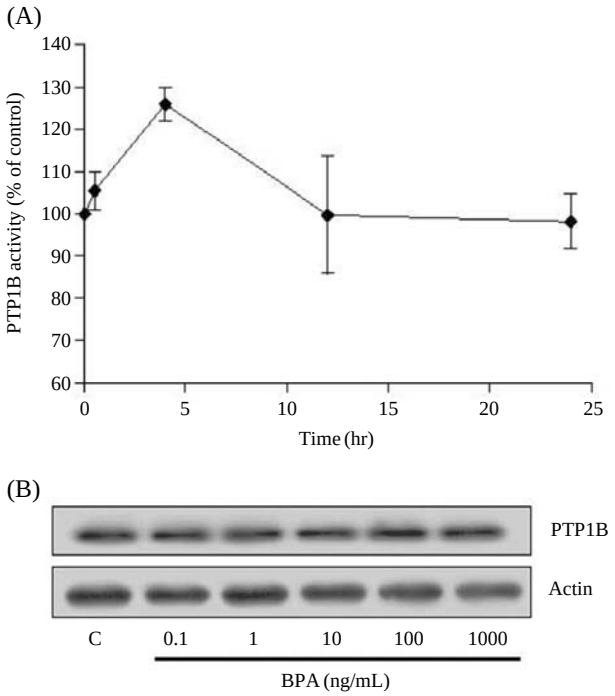

Figure 5. Effect of BPA on PTP1B activity. C2C12 cells were treated with BPA (100 ng/mL) for various times (0.5, 4, 12, and 24 h) (A). PTP1B activities were determined by measuring the hydrolysis of p-nitrophenyl phosphate (pNPP) (B). Western blots of PTP1B after treatment with BPA (0.1-1,000 ng/mL) for 24 hours.

shown to increase BPA leaching rates[11].

Recent studies have demonstrated that BPA affects insulin secretion and glucose metabolism[12,13]. Furthermore, the hyperinsulinemia produced by BPA affects peripheral tissues and causes insulin resistance, probably by down-regulating insulin receptor numbers and function[14]. In this study, we examined the effect of BPA on FBG in post-weaning Wistar rats. Seven days of BPA treatment (0.5 μg/μL/h continuously infusion by osmotic minipumps) increase FBG and insulin plasma levels. In this study, we administered BPA using a subcutaneous osmotic pump to these rats. Although, routes of BPA administration are important, it has been reported that non-oral routes of administration, in fetuses and neonates both the enzyme that conjugate BPA is expressed at low levels which suggests that the pharmacokinetics of oral and non-oral dosing are similar[15].

However, the effects of BPA on insulin signaling molecules in skeletal muscle have not been previously investigated. In the present study, the effects of BPA were also determined using myotubes that were differentiated from mouse skeletal muscle C2C12 cells. For most cells, glucose homeostasis is achieved via the insulin dependent translocation of the glucose transporter, GLUT4, from intracellular vesicles to the cell surface[16-18]. Insulin signal transduction in skeletal muscle is mediated by a series of phosphorylation cascades that link the initial activation of insulin receptor (IR) tyrosine kinase to downstream substrates. Insulin-receptor substrates (IRS-1 to 4) are regulatory docking proteins that associate with IR and play a central role in the selection and direction of insulin signals toward further metabolic or gene regulatory events.

Our study shows that insulin-mediated Akt phosphorylation was inhibited by BPA. The mRNA expression of IR in C2C12 cells was reduced after 24 hours of BPA treatment in a dose-dependent manner, whereas the mRNA expression of IRS-1 was unaffected. Hypoxia and 5-aminoimidazole-4-carboxamide 1-β-D-ribonucleoside (AICAR) increases glucose transport via an insulin-independent mechanism involving AMPK activation. However, BPA did not affect AMPK expression in C2C12 myotubes.

We also found that treating myotubes with BPA increased PTP1B activity, and PTP1B levels in muscles and adipose tissues have been reported to be correlated with degree of insulin resistance in subjects with diabetes and obesity[19,20]. In the present study, PTP1B increased rapidly after 5 hours of BPA treatment and then decreased slowly to return to baseline at between 12 and 24 hours. BPA treatment also increased fasting blood glucose and plasma insulin levels, which induced the overexpression of PTP1B and decreased IR signaling and insulin resistance in rapid response. In addition, BPA increased GLUT4 expression in C2C12 myotubes. These results suggest that the BPA-induced inhibition of insulin signaling drives the compensatory up-regulation of GLUT4.

Consequently, exposure to BPA can induce insulin resistance via mechanisms that include decreased IR gene expression and increased PTP1B activity. Additional studies are required on the effects of BPA during early development, the neonatal period, and in childhood to evaluate whether chronic BPA exposure can lead to insulin resistance development and type 2 diabetes.

Materials & Methods

Chemicals and Cell Culture

Bisphenol A (BPA) (Fluka, Buchs, Switzerland); p-nitrophenyl phosphate (pNPP) (Sigma, MO, USA); polyclonal anti-phospho-Akt (ser473) and anti-Akt antibodies (Cell Signaling Technology, MA, USA);

and polyclonal anti-PTP1B antibody (Upstate Biotechnology, INC., NY, USA) were purchased commercially. Mouse myoblast C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) and penicillin/streptomycin at 37˚C in a 5% CO_2 atmosphere. Confluent cells were differentiated in DMEM containing 2% horse serum and fused into myotubes after 4-5 days.

Animals and Treatment

Three week-old male Wistar rats were housed individually in a temperature (22±2˚C) and humidity (30-40%) controlled room under a 12 h light/dark cycle with free access to food and water. Animals were randomly divided to a control group (n=7) and a BPA-treated experimental group (n=8). The experimental group was administered BPA continuously for 7 days using osmotic mini-pump (Alzet model 1007D, 0.5 μL/hr, Alzet Corporation, Palo Alto, CA), which was implanted subcutaneously. For the control group, propylene glycol was infused using a mini-osmotic pump in a similar manner. Blood glucose was measured using a Gluco Dr glucometer (Handi Co., Ltd., Incheon, Korea) and plasma insulin was assayed using a Rat Insulin ELISA KIT (SHIBAYAGI, Co., Ltd., Shibukawa, Gunma, Japan).

Western Blot Analysis

After electrophoresis, proteins were transferred to PVDF membranes. Membranes were blocked with 5 % nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 2 hours at room temperature and incubated with an anti-phospho-Akt (Ser473) (1 : 1,000), anti-Akt (1 : 1,000), or anti-PTP1B (1 : 1,000) antibody overnight at 4˚C. Membranes were then briefly incubated with a Pico-signal ECL system (Pierce) and exposed to X-ray films. Films were scanned and analyzed using Bio-Profil Bio1D software.

Measurement of PTP1B Activity

Immunoprecipitates were collected, pNPP was hydrolyzed at 37˚C for 1 h, and the reaction was then quenched by adding 100 μL of 1 M NaOH. The amounts of p-nitrophenyl formed were measured by determining absorbance at 405 nm using a spectrophotometer.

RNA Isolation and RT-PCR

RNA was quantified by measuring absorbance at 260 nm. RT-PCR was performed to assay the expressions of the AMPK, IR, IRS-1, and GLUT4 genes in C2C12 myotubes. PCR products were resolved electrophoretically in 1% agarose gels (w/v; NuSieve 1 : 1, FMC Bioproducts, Rockland, ME, USA) containing 1 μg/mL ethidium bromide, and visualized under a UV transilluminator.

Statistical Analysis

All data are presented as means±SE. The effects of treatments were compared using the Student's *t*-test in GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA). *P* values of <0.05 were considered statistically significant.

Acknowledgements

This work was supported by grant from Inje University, 2006.

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