Toxicology Letters 209 (2012) 78–85

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Regulation of adiponectin secretion by soy isoflavones has implication for endocrine function of the testis

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form
25 November 2011
Accepted 26 November 2011
Available online 3 December 2011

Keywords:
Isoflavones
Phytoestrogens
Adipose tissue
Adiponectin
Leydig cells
Androgen

ABSTRACT

Testicular Leydig cells are the predominant source of the male sex steroid hormone testosterone (T), which is required to maintain male fertility. There is now growing evidence that environmental stressors, including chemicals present in food, air and water, may affect energy balance. A relationship between energy balance and reproductive capacity has been proposed for a long time. In the present study, developmental exposures of male rats to soy isoflavones in the maternal diet from gestational day 12 to day 21 post-partum enhanced adiponectin expression in adipose tissue and increased serum adiponectin concentrations in adulthood. However, exposure to soy isoflavones caused a decrease in T production and expression of adiponectin and its receptor (adipoR2) in Leydig cells. In separate experiments, incubation of Leydig cells with recombinant adiponectin in the absence of isoflavones caused a decrease in T biosynthesis associated with diminished expression of the cholesterol transporter steroidogenic acute regulatory protein (StAR). Thus, chemical-induced alterations in serum adiponectin concentrations have implication for steroid hormone secretion. The results also imply that changes in adipose tissue metabolism occasioned by exposure to dietary estrogens, and perhaps other estrogenic agents, possibly contribute to deficiencies in reproductive capacity attributed to these compounds.

1. Introduction

During development, the perinatal environment plays an important role in programming several aspects of physiology and behavior. Also, body energy reserves and metabolic states are thought to regulate pubertal development and fertility. Therefore, it is not surprising that metabolic derangements, including energy insufficiency and obesity, are linked to reproductive disorders (Sullivan et al., 2011). A growing body of evidence now proposes that environmental chemicals with hormone-like activity, which are present in the air, food or water, can disrupt programming of endocrine signaling pathways during development and cause adverse effects in adulthood, including obesity and diabetes (Heindel, 2003). Indeed, it has been suggested that the obesity epidemic in the population coincided with the marked increase of industrial chemicals in the environment over the past 40 years (Baillie-Hamilton, 2002). Although there has been long standing evidence that gonadotropin releasing hormone (GnRH) secretion by the hypothalamus and/or gonadotropin release from the pituitary gland are disrupted in altered metabolic states (Koch et al., 2008; Cederroth, 2009), much of the mechanisms governing the association between energy balance and reproduction remain to be clarified.

Acting through a network of autocrine, paracrine and endocrine pathways, adipokines, i.e., factors secreted by adipose tissue, are involved in the regulation of several aspects of physiology, including glucose and lipid metabolism, neuroendocrine function, reproduction, immunity, and cardiovascular function (Shankar et al., 2010; Pataky et al., 2010). For example, adiponectin, a 30 kDa protein, is associated with the regulation of insulin sensitivity (Kershaw, 2004). The levels of serum adiponectin are altered in multiple metabolic conditions and adiponectin is known to exert its action by binding to two receptor isoforms (adipoR1 and adipoR2). Adiponectin receptors have seven putative transmembrane domains but are functionally distinct from G-protein-coupled receptors (Caminos et al., 2008). Interestingly, testicular Leydig cells, the predominant source of the male sex steroid hormone testosterone (T) that supports male reproductive activity, express adiponectin receptors (adipoR2) (Caminos et al., 2008). However, little is known about the role of adipose tissue-derived factors in the regulation of testis function.

There is evidence indicating that genistein, one of two predominant isoflavones present in soybeans (the other is daidzein), affects adipose tissue development in a dose-dependent and

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doi:10.1016/j.toxlet.2011.11.027

gender specific manner (Penza et al., 2006). Regulation of adipose tissue metabolism by soy isoflavones has relevance to public health because soy-based products have become increasingly popular in the diet for their putative health beneficial effects, including prevention of diabetes. However, it remains to be clarified that metabolic molecules, e.g., adiponectin, act directly in testicular cells. In the present study, we determined whether developmental exposures to isoflavones affect adiponectin secretion at sexual maturity in the male rat and if adiponectin can interfere with the androgen biosynthetic pathway.

2. Materials and methods

2.1. Animal studies

The experimental protocol was described previously (Sherrill et al., 2010). Briefly, time-bred female Long–Evans rats ($n = 14$ per group) were allowed to acclimate in the housing facility at Auburn University's College of Veterinary Medicine Laboratory Animal Facility for three to four days before commencement of experiments. The Long–Evans strain of rats was used in these studies due to concerns that the Sprague-Dawley strain, typically used in toxicological studies, is not sensitive to some estrogenic chemicals (Steinmetz et al., 1997; Ben-Jonathan et al., 2000). Animals were maintained under constant conditions (12 h daylight, 12 h darkness) and temperature between 20 °C and 23 °C and with free access to pelleted food and water in glass bottles. The concentrations of isoflavones in the experimental diets were 0 ppm, 5 ppm, 50 ppm, or 1000 ppm based on the assayed content of genistin and daidzin by the manufacturer (Harlan-Teklad, IN). Pregnant dams were fed diets from gestational day (GD) 12 to postnatal day (PND) 21 and the date of birth was designated as PND 1. Male rats were exposed to different isoflavone levels in the maternal diet only for the perinatal period (GD 12 to PND 21), whereas animals from all groups were fed the soy-free control diet from PND 21 to 90. The levels of isoflavones (free aglycones) in weanling male offspring at 21 days of age, i.e., within 24 h of termination of isoflavone exposure, were not measured in the present study but were determined to be ~70 nM for genistein and ~105 nM for daidzein in a previous study using the same diet formulations (Akingbemi et al., 2007). In the present study, blood was collected from a cohort of 8–10 animals per diet group at 90 days of age to analyze serum biochemistry. In addition, gonadal fat and Leydig cells were isolated from the same animals and stored at −80 °C until processed for SDS-PAGE and Western blot analyses. The levels of estrogen receptors types 1 and 2 (ESR1, ESR2) and adiponectin were analyzed in gonadal fat, whereas adiponectin and adipoR2 protein and T production were measured in Leydig cells.

2.2. In vitro experiments

Leydig cells isolated from 21- and 35-day old rats were used in in vitro experiments because these are developing stages of Leydig cell development, possessing characteristics of prepubertal and fully differentiated adult Leydig cells (Benton et al., 1995). The experiments were designed to investigate the relationship between adiponectin concentrations and Leydig cell function. Therefore, Leydig cells were incubated in DMEM/F-12 culture media buffered with 14 mM $NaHCO_3$ and 15 mM HEPES (Sigma–Aldrich, St. Louis, MO, USA) and containing 0.1% bovine serum albumin (BSA) (MP Biomedicals, Solon, OH, USA) and 0.5 mg/ml bovine lipoprotein in 6-well plates (1.0×10^6 cells/well) for an initial period of 1 h to acclimate and then in media containing recombinant adiponectin (0 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml) (R&D Systems, Minneapolis, MN, USA). Incubations were performed at 37 °C and 5% CO_2 under humidified conditions for 18 h. In order to exclude the possibility of changes in the number of Leydig cells during the incubation period, cells were harvested after treatment and incubated in fresh media without (basal) and with ovine LH (LH-stimulated) at 34 °C for 3 h. Ovine LH was obtained from the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA).

Although the present study is focused on adipokine regulation of Leydig cells, we nevertheless performed experiments to determine whether insulin acts differently than adiponectin in Leydig cells. Insulin plays a major role in conditions of altered metabolic homeostasis and is present in serum at elevated levels in most diabetic states (Thompson and Regnault, 2011). Therefore, Leydig cells were incubated in DMEM/F-12 culture media containing insulin (0 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml) for 24 h. In all cases, aliquots of spent media at the end of treatment were kept at −20 °C until analyzed by RIA and T secretion was normalized to ng/10^6 cells. Control, adiponectin- and insulin-treated Leydig cells were processed to obtain lysates for Western blotting to analyze the levels of protein regulators of Leydig cell differentiated function, e.g., proliferating cell nuclear antigen (PCNA), luteinizing hormone receptors (LHR), StAR and protein kinases (e.g., protein kinase B or AKT and extracellular regulated kinase or ERK) (Sherrill et al., 2010).

2.3. Serum biochemistry

Adult male rats at 90 days of age that were exposed to isoflavones in the maternal diet during the perinatal period (GD 12 to PND 21) were fasted overnight. In the morning, blood was collected at sacrifice by CO_2 asphyxiation and separated to obtain serum for measurement of glucose, insulin, leptin and adiponectin concentrations. Glucose concentrations were measured using a Glucose Assay kit (Biovision, Mountain View, CA, USA). Insulin and adiponectin concentrations were measured by ELISA using rat insulin or adiponectin as standard (Linco Research Immunoassay, St. Charles, MO, USA). For the insulin assay, the interassay and intraassay coefficients of variation were 7.4% and 4.7%, respectively, and assay sensitivity was 0.2 ng/ml. For the adiponectin assay, interassay and intraassay coefficients of variation were 6.54% and 1.59%, respectively, while sensitivity was 0.155 ng/ml. Serum leptin was measured using an ELISA kit and rat leptin as standard (Millipore Corporation, St. Charles, MO, USA). The interassay and intraassay coefficients of variation were 2.95% and 2.13%, respectively while assay sensitivity was 0.4 ng/ml.

2.4. Isolation of Leydig cells

Purified Leydig cells were obtained from 21-, 35- and 90-day old male rats by collagenase digestion of the testis followed by loading of the cell suspension onto a 55% continuous Percoll density gradient and centrifugation at $12{,}000 \times g$ according to the method of Klinefelter (Klinefelter and Ewing, 1988). Developing Leydig cells (PND 21 and 35) were harvested from the Percoll gradient at a band between 1.070 and 1.088, and Leydig cells obtained on PND 90 at 1.070 and greater. Cell yields were estimated with a hemocytometer, and purity was assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD), using 0.4 mM etiocholan-3β-ol-17-one as the enzyme substrate (Payne et al., 1980). Leydig cell fractions were typically 95–97% enriched for cells that stained intensely for the marker enzyme.

2.5. Western blot analysis

Gonadal fat and Leydig cells were homogenized in buffer containing a protease inhibitor cocktail, including 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 0.5 M EDTA, 2 mM leupeptin, and 1 mM peptatin (# 78410, Pierce Biotechnology, Inc., Rockford, IL, USA). Following centrifugation at $15{,}000 \times g$ for 20 min at 4 °C, the supernatant was collected and stored at −80 °C. Aliquots of supernatants were assayed for protein content by the Bradford assay and subsequently electrophoresed through 10% SDS–PAGE and transferred to nitrocellulose membranes (0.45 μm). Membranes were blocked with 5% milk in PBS–Tween-20 buffer (Bio-Rad Laboratories, Philadelphia, PA, USA). After blocking, membranes were washed and incubated with primary antibodies overnight at 4 °C. All antibodies were sourced from Santa Cruz Biotechnology (Santa Cruz, CA, USA) [i.e., ESR2 (goat polyclonal, sc-6821), LHR (sc-25828), (StAR) (sc-25806), PCNA (sc-53409), AKT (sc-8312), phosphorylated-AKT or $pAKT^{Ser473}$ (sc-7985), ERK (sc-93), phosphorylated ERK or p-ERK (sc-7383)] except for ESR1 (ab 2746–50, Abcam, Cambridge, MA, USA) and phosphorylated StAR protein phosphorylated on serine residue 194 (Dr. Steve King, Baylor College of Medicine, Houston, TX, USA), adiponectin (# 5903-50, Biovision, Mountain View, CA, USA) and adipoR2 (PA1-1071, Affinity Bioreagents, Rockford, IL, USA).

Table 1
Body weights and serum concentrations of glucose, insulin and leptin in adult male rats exposed to soy isoflavones in maternal diet during the perinatal period (GD 12 to PND 21).

	Isoflavone levels in maternal diet (ppm)			
	0	5	50	1000
Body weights (PND 21)	62 ± 0.9	60 ± 0.8	62 ± 1.3	60 ± 1
Body weights (PND 90)	479 ± 11	438 ± 17	429 ± 11	422 ± 13
Serum glucose (ng/ml)	140 ± 9	170 ± 19	165 ± 8	157 ± 13
Serum insulin (ng/ml)	3.3 ± 0.4	3.5 ± 1.8	3.7 ± 0.7	4.0 ± 0.5
Serum leptin (ng/ml)	7.8 ± 0.8	10.7 ± 2.4	12.5 ± 2*	11.5 ± 2

GD, gestational day; PND, postnatal day.
* Significantly different from control ($P < 0.05$).

Blots were washed to remove unbound antibodies before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The presence of the appropriate proteins was visualized using a chemiluminescent horseradish peroxidase antibody detection reagent followed by exposure to autoradiography films (HyGLO; Denville Scientific Inc., Metuchen, NJ, USA). Protein expression levels were quantified using the Epson 4180 Perfection scanning software (Epson-America, Long Beach, CA, USA). Proteins in immunoblots were measured as optical density of the bands on exposed Autorad films using Doc-lt LS software (Ultra-Violet Products Ltd., Upland, CA, USA). To measure adiponectin and adipoR2 protein, membranes were incubated overnight at 4 °C in primary antibody after blocking nonspecific binding with the Odyssey blocking buffer (LI-Cor Biosciences, Lincoln, NE, USA). Subsequently, infrared labeled secondary antibodies were added to bind to the primary antibody. The bound complex was detected using the Odyssey Infrared Imaging System (LI-Cor Biosciences; Lincoln, NE, USA). Phosphorylated proteins (p-AKT, p-ERK) were normalized to corresponding total or inactive protein levels, whereas other proteins (ESR1, ESR2, PCNA, LHR, StAR, adiponectin and adipoR2) were normalized to β-actin.

2.6. Statistics

Data are described as mean ± SD. Adult male rats at 90 days of age that were perinatally exposed to soy isoflavones were randomly selected from each diet group for analysis (n = 8–10). Experiments in vitro were performed using cell preparations obtained on three different occasions, and each cell isolation procedure involved collecting testes from thirty five 21 day-old and eighteen 35 day-old male Long–Evans rats. Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple group comparisons (GraphPad, Inc., San Diego, CA). Differences of $P < 0.05$ were considered significant.

3. Results

3.1. Body weights and serum glucose and insulin concentrations

Body weights at 21 and 90 days of age were not affected by exposure to isoflavones in maternal diet from GD 12 to PND 21 and are shown in Table 1. The serum levels of glucose and insulin were also similar in control and isoflavone-exposed animals (Table 1) ($p > 0.05$).

3.2. Isoflavones increased serum estradiol levels and decreased ESR protein expression in adipose tissue

Serum estradiol concentrations were increased in male rats exposed to the higher levels of isoflavones in maternal diet at 50 and 1000 ppm in the perinatal period, i.e., GD 12 to PND 21, compared to control (Fig. 1A) ($P < 0.05$). In contrast, the levels of ESR1 protein were decreased in adipose tissue from the 5 and 1000 ppm diet groups, whereas ESR2 protein was reduced only in the 1000 ppm group compared to control ($P < 0.05$) (Fig. 1B and C).

3.3. Isoflavones increased serum adiponectin and leptin concentrations and increased adiponectin protein in adipose tissue

Serum adiponectin and leptin concentrations were greater in male rats exposed to isoflavones in the maternal diet at 50 ppm during the perinatal period (GD 12 to PND 21) compared to control (Fig. 2A and, Table 1) ($P < 0.05$). Similarly, adiponectin protein was present at greater levels in adipose tissue from adult male rats exposed to isoflavones in the perinatal period compared to control and the greatest levels were seen in the 50 ppm diet group (Fig. 2B) ($P < 0.05$).

3.4. Expression of adiponectin receptors is developmentally regulated in Leydig cells and was decreased along with adiponectin protein and androgen secretion by isoflavones

Receptors for adiponectin (adipoR2) were present at all stages of Leydig cell development and levels were greater in the pubertal (PND 35) and adult period (PND 90) than in neonatal rats (PND 21)

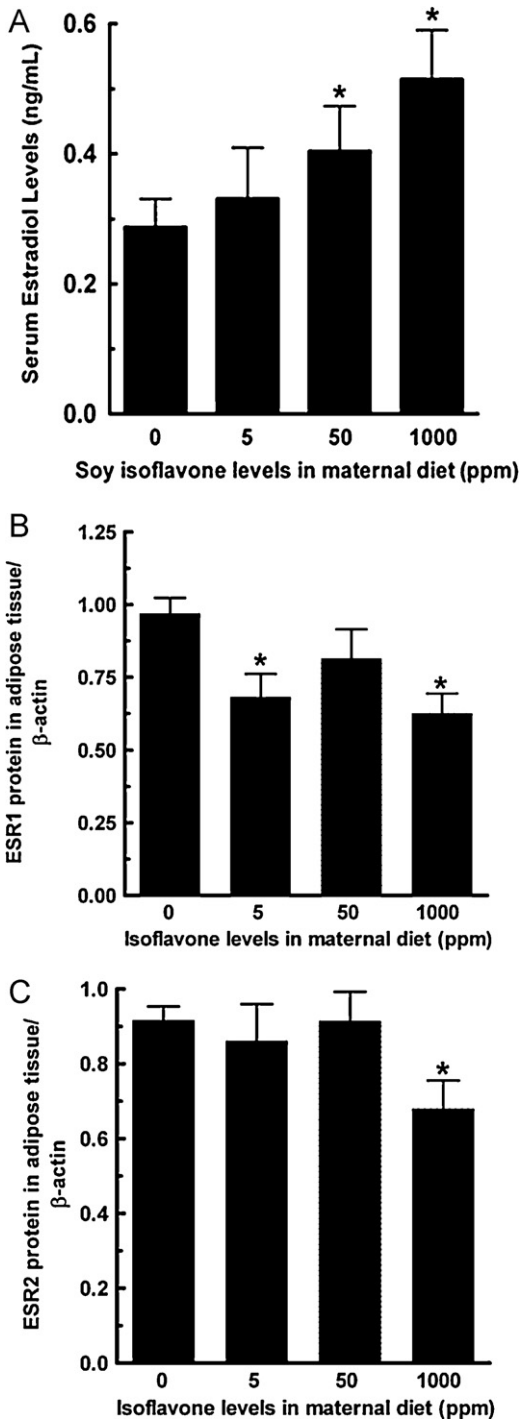

Fig. 1. After exposure of male rats to soy isoflavones in maternal diet from gestational day 12 to postnatal day 21, serum and gonadal fat were obtained from 8 to 10 male rats per group at 90 days. Serum was analyzed for estradiol concentration by RIA, whereas estrogen receptor (ESR1, ESR2) protein levels in gonadal fat were measured using Western blotting procedures and normalized to β-actin as internal control. Data are described as mean ± SD. Values with asterisks were significantly different from control (*$P < 0.05$).

(Fig. 3A) ($P < 0.05$). Moreover, exposure of male rats to isoflavones in the perinatal period, i.e., GD 12 to PND 21, caused a decrease in adiponectin and adipoR2 protein in Leydig cells at 90 days of age compared to control (Fig. 3B and C) ($P < 0.05$). Similarly, Leydig cell T secretion was decreased in the 1000 ppm diet group compared to control (Fig. 3D) ($P < 0.05$).

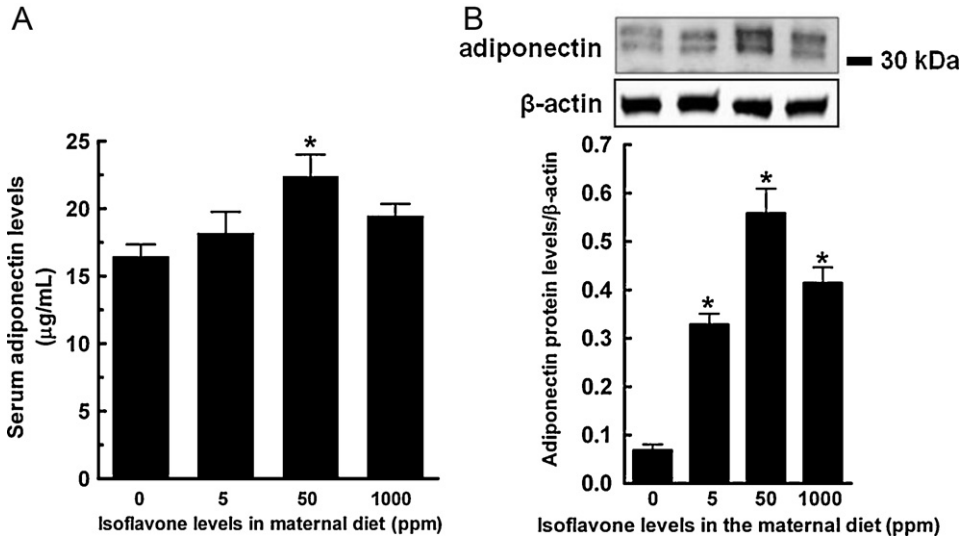

Fig. 2. After exposure of male rats to soy isoflavones in maternal diet from gestational day 12 to postnatal day 21, serum was separated from blood obtained at 90 days and analyzed for adiponectin concentrations by ELISA (A), whereas adiponectin protein was measured in Western blots of gonadal fat (B). Adiponectin protein was normalized to β-actin as internal control. Serum and gonadal fat were analyzed in 8–10 animals per group. Adiponectin, 30 kDa; β-actin, 42 kDa. Data are described as mean ± SD. Values with asterisks were significantly different from control (*$P < 0.05$).

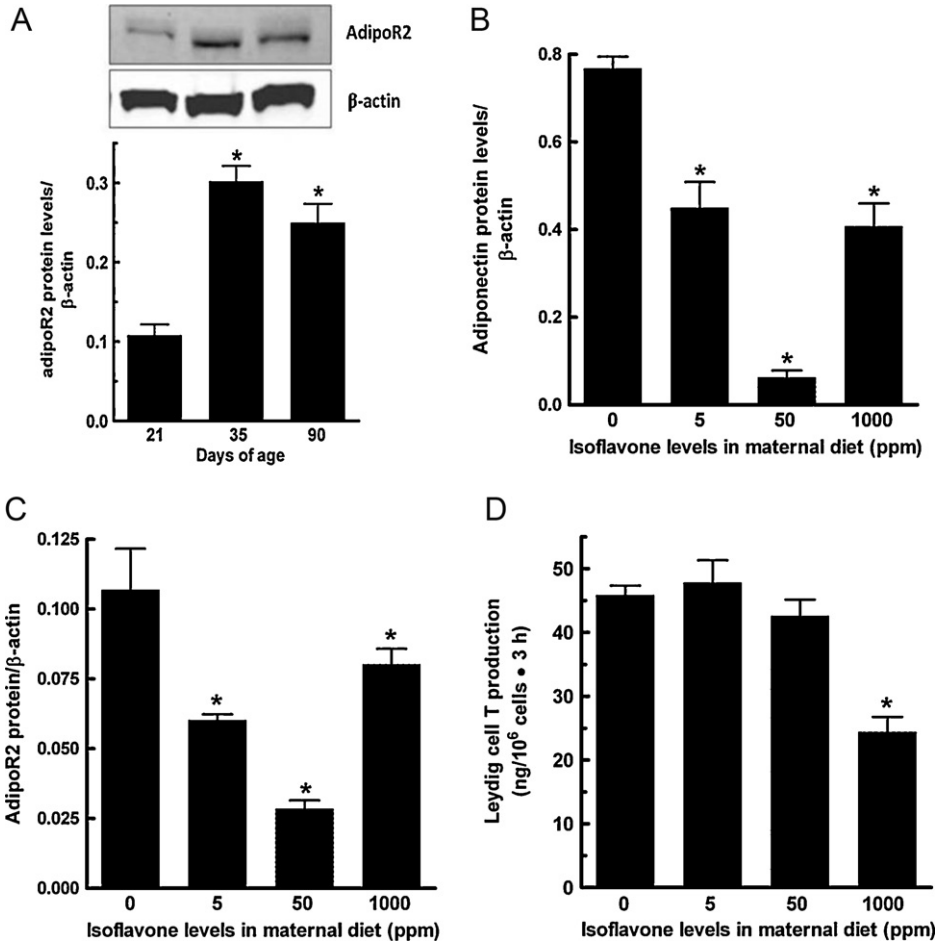

Fig. 3. Expression of adiponectin receptor protein (adipoR2) was analyzed in progenitor (21 days), immature (35 days) and adult Leydig cells (90 days) isolated from male rats not previously exposed to soy isoflavones (A). In separate experiments, adiponectin and adipoR2 protein were analyzed in adult Leydig cells (90 days) after exposure of male rats to soy isoflvaones in maternal diet from gestational day 12 to postnatal day 21 (B, C). Also, Leydig cells obtained at 90 days were incubated in culture media for 3 h and the concentrations of T in spent media were measured by RIA (D). Adiponectin and adipoR2 protein were analyzed using Western blotting procedures, which were performed four to five times and were normalized to β-actin as internal control. Adiponectin, 30 kDa; adipoR2, 43 kDa; β-actin, 42 kDa. Data are described as mean ± SD. Values with asterisks were significantly different from control (*$P < 0.05$).

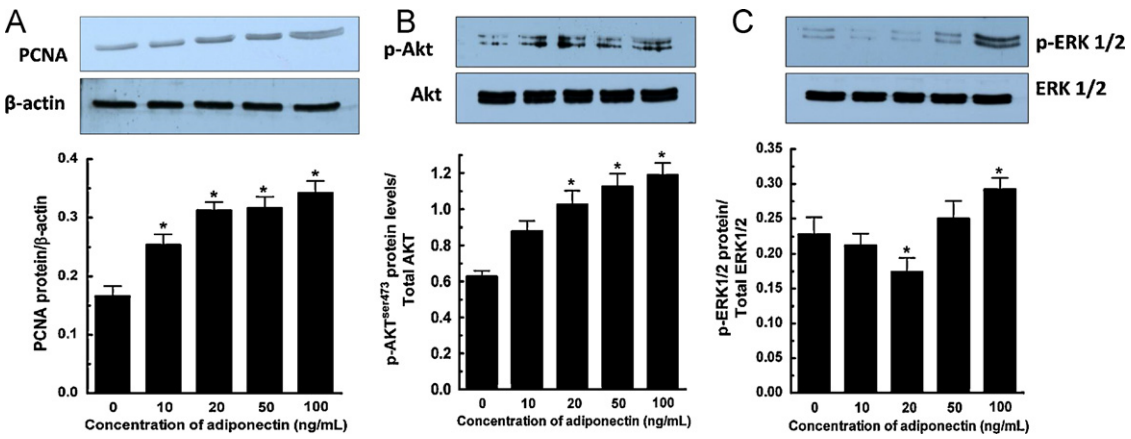

Fig. 4. Leydig cells were isolated from three separate groups of thirty five 21-day old male rats and incubated in media containing recombinant adiponectin for 18 h ($n = 3$). After treatment, Leydig cell lysates were processed for Western blot analysis using primary antibodies specific to proliferating cell nuclear antigen (PCNA) (A) and protein kinase B (AKT) (B) and extracellular regulated kinase (ERK) (C). Figures represent results from three independent experiments. Western blotting procedures were performed three to five times using Leydig cell lysates from each experiment. PCNA, 36 kDa; AKT/p-AKT^{ser478}, 56/60 kDa; ERK/p-ERK, 42/44 kDa. Data are described as mean ± SD. Values with asterisks were significantly different from control (*$P < 0.05$).

3.5. Adiponectin induced cell cycle protein expression and regulated protein kinase activation in Leydig cells

Incubation of Leydig cells with recombinant adiponectin caused greater expression of the cell cycle protein PCNA compared to control (Fig. 4A) ($P < 0.05$). In addition, adiponectin treatment affected activation of protein kinases AKT and ERK in Leydig cells as indicated by greater ratios of phosphorylated proteins to the corresponding total or inactive protein compared to control (Fig. 4B and C) ($P < 0.05$).

3.6. Adiponectin acted directly in Leydig cells to suppress androgen biosynthesis

Incubation with recombinant adiponectin caused a dose-dependent decrease both in the levels of basal and LH-stimulated T secretion by Leydig cells compared to control (Fig. 5A and B) ($P < 0.05$). Results of Western blot analysis showed that adiponectin treatment of Leydig cells at 100 ng/ml caused a decrease in StAR protein, but not LHR, compared to control (Fig. 5C and D) ($P < 0.05$).

3.7. Insulin caused an inhibitory effect on androgen biosynthesis

Incubation with insulin caused a concentration-dependent inhibition of T production by Leydig cells compared to control as shown in Fig. 6A ($P < 0.05$). Suppression of androgen biosynthesis by insulin was associated with a decrease in the rate of phosphorylation of the StAR protein (p-StAR) compared to control (Fig. 6B) ($P < 0.05$).

4. Discussion

The present results demonstrated that exposures to dietary estrogens during development may cause metabolic changes that affect adipokine secretion. Although body weights were similar, isoflavones caused a decrease in ESR expression in adipose tissue. In contrast, developmental exposure to isoflavones increased adiponectin protein in adipose tissue, which corresponded to elevated serum adiponectin concentrations. The possibility that adiponectin regulates Leydig cells was indicated by the presence of adiponectin protein at all stages of Leydig cell development. Indeed, the amounts of adiponectin and adipoR2 protein and T production by Leydig cells were lower than control in adult male rats that were perinatally exposed to isoflavones.

Our observation of a pubertal increase in adipoR2 protein in Leydig cells is similar to previous reports in the rat (Caminos et al., 2008), mouse (Combs et al., 2003) and chicken testis (Ocón-Grove et al., 2008). However, the finding of abundant adipoR2 protein in Leydig cells at 35 days is at variance with low mRNA levels seen at 30 days (Caminos et al., 2008). The disparity in adipoR2 mRNA and protein may be due to changes in the rate of transcriptional and/or translational activity during the 5-day interval or is the result of decreased degradation of the adipoR2 protein, which has the effect of increasing its half-life (Leppek et al., 2011). Altogether, these findings are relevant to public health because both adipoR1 and adipoR2 are expressed in the human testis (Civitarese et al., 2004).

Results of in vitro experiments showed that adiponectin acted directly in Leydig cells to decrease androgen secretion, which was associated with inhibition of the StAR protein in Leydig cells. The cholesterol transporter StAR is required for translocation of the cholesterol substrate into mitochondria for the first enzymatic action occurring during androgen biosynthesis (Manna et al., 2009). Considered with previous observations showing that adiponectin has the capacity to act in the pituitary and cause changes to gonadotropin regulation of the testis (Rodriguez-Pacheco et al., 2007), our results imply that metabolic factors have the potential to exert their effects on gonadal function at multiple levels of the hypothalamic–pituitary–gonadal axis.

A relationship between steroid hormone action and adipose tissue development was described previously (Cooke and Naaz, 2005). For example, evidence that estrogen is a major regulator of adipogenesis was provided by the finding of greater fat mass in male mice that were lacking ESR1 or the aromatase gene (Heine et al., 2000; Jones et al., 2000). Similarly, men lacking either aromatase or ESR1 have a tendency towards obesity and insulin resistance (Maffei et al., 2004). On the other hand, gonadectomy was found to decrease adiponectin levels in adult male mice (Combs et al., 2003), whereas the high concentrations of serum adiponectin seen in hypogonadal men were reduced by androgen supplementation (Lanfranco et al., 2004). Taken together, these observations suggest that a reciprocal relationship exists between gonadal steroid hormones and adipose tissue-derived factors. Thus, decreased adipose tissue expression of the ESR protein seen in the present study has the potential to affect adipose tissue function and possibly contribute to changes in adiponectin gene expression.

The present data showed that developmental exposure to isoflavones increased serum adiponectin and leptin levels in adult male rats. This finding is intriguing because there are obvious

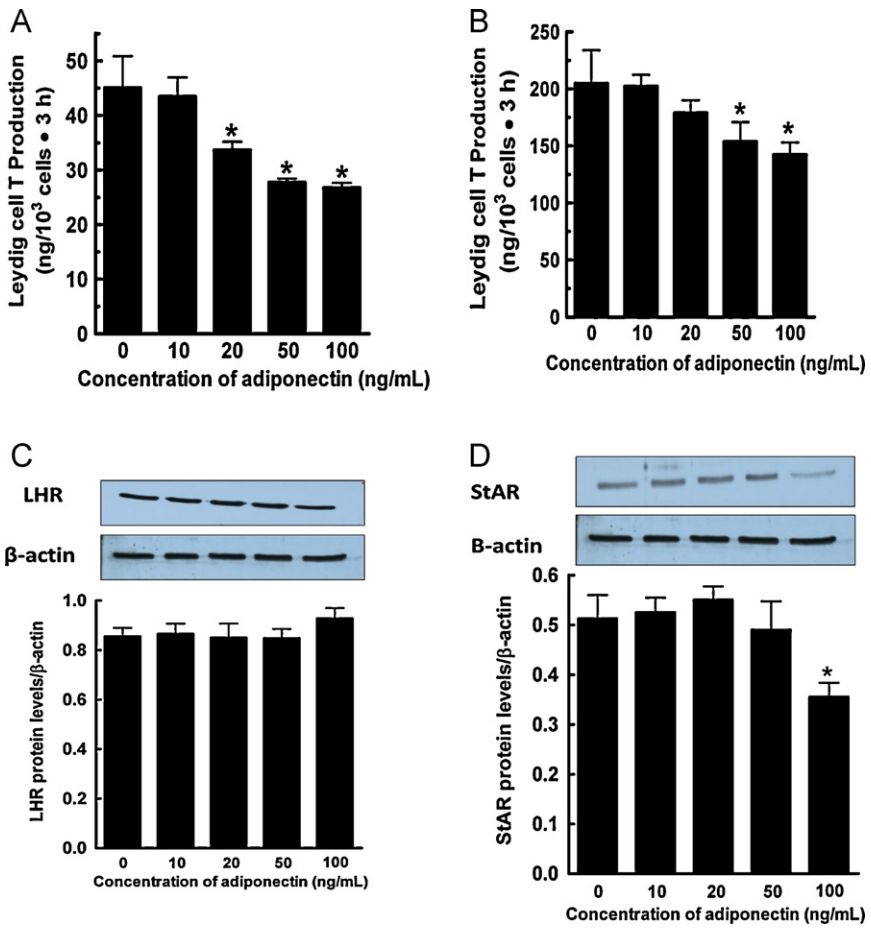

Fig. 5. Leydig cells were isolated from three separate groups of eighteen 35 day-old male rats and incubated in culture media containing recombinant adiponectin for 18 h ($n = 3$). Cells were harvested and incubated further in adiponectin-free media without (basal, A) or with ovine luteinizing hormone (LH) for 3 h (LH-stimulated, B). The amounts of testosterone (T) produced by Leydig cells were measured in aliquots of spent media by RIA. Also, control and adiponectin-treated Leydig cells were processed for western blot analysis to measure luteinizing hormone receptor (LHR) and steroidogenic acute regulator (StAR) protein (C, D). Figures represent results from three independent experiments and western blotting was performed at least three to five times on each occasion. Protein levels were normalized to β-actin as internal control. LHR, 80 kDa; StAR, 30 kDa; β-actin, 42 kDa. Data are described as mean ± SD. Values with asterisks were significantly different from control (*$P < 0.05$).

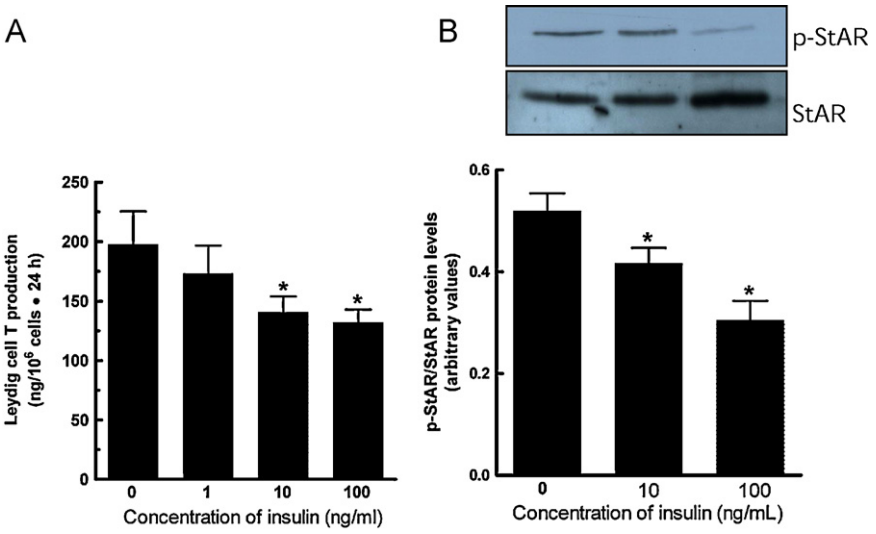

Fig. 6. Leydig cells were isolated from three separate groups of eighteen 35-day old male rats and incubated in media containing insulin for 24 h ($n = 3$). The amounts of testosterone (T) in aliquots of spent media were measured by RIA (A), whereas steroidogenic acute regulatory (StAR) and phosphorylated StAR (p-StAR) levels were analyzed in Western blots (B). Figures represent results from three independent experiments. Western blotting was performed three to five times using Leydig cell lysates from each experiment. The levels of p-StAR protein were normalized to total StAR protein. StAR/p-StAR, 30 kDa. Data are described as mean ± SD. Values with asterisks were significantly different from control (*$P < 0.05$).

differences in the pathophysiological features of the two adipokines. For example, adiponectin levels, unlike leptin, are decreased in obese subjects in comparison with lean controls and are increased concomitantly with weight loss (Diez and Iglesias, 2003; Faraj et al., 2003). Also, adiponectin is thought to reduce body weight by increasing energy expenditure rather than decreasing food intake (Brooks et al., 2007), whereas leptin acts centrally in the hypothalamus to suppress appetite while increasing insulin sensitivity and energy expenditure in peripheral tissues (Schwartz et al., 2000; Muoio and Lynis Dohm, 2002). Despite reports of dissimilar mechanisms of action, there seems to be a general consensus that adiponectin and leptin both increase insulin sensitivity (Carnier et al., 2011). Therefore, the present findings are aligned with the view that consumption of soy-based diets may alleviate pathophysiological signs associated with metabolic syndromes (Mohamed Salih et al., 2009). However, isoflavone effects may be dose-dependent because greater serum adiponectin and leptin concentrations were measured in adult rats from the 50 ppm diet group than in the 5 and 1000 ppm groups. Thus, additional studies are required to characterize the relationship between isoflavone exposure levels and adipokine concentrations.

Previous reports determined that adipoR1 is localized to the seminiferous epithelium, whereas adipoR2 is expressed in interstitial Leydig cells and may serve to link metabolic homeostasis and testis function (Bjursell et al., 2007; Caminos et al., 2008). Our observation that adiponectin inhibits androgen secretion and activated protein kinases is similar to previous reports in the rat showing that adiponectin inhibited basal and human chorionic gonadotropin-stimulated T secretion in the adult testis (Caminos et al., 2008) and induced AKT and ERK1/2 phosphorylation in the ovary (Chabrolle and Dupont, 2007; Pierre et al., 2009). However, Leydig cell T secretion was inhibited by adiponectin at concentrations equal to or greater than 20 ng/ml but StAR protein was decreased only at 100 ng/ml. Although not analyzed in the present study, it is possible that steroidogenic enzyme gene expression was affected at the 20 ng/ml and 50 ng/ml concentrations and contributed to deficits in androgen biosynthesis. Nevertheless, these observations imply that adiponectin has the capacity to regulate steroid hormone secretion.

The finding of a nonlinear effect, i.e., lack of a dose–response or monotonic effect, on several parameters (e.g., ESR1 and adiponectin protein in adipose tissue, and adipoR2 protein and ERK phosphorylation in Leydig cells), is perhaps not surprising. This phenomenon has been previously and widely attributed to the action of several estrogenic compounds and endocrine disruptors (Welshons et al., 2003) and affects biological parameters in a tissue-dependent manner (Vandenberg et al., 2006). Moreover, the non-monotonic or U-shaped or inverted U-shaped response, seen in this study, appears to be typical of receptor-mediated responses, which saturate and become alleviated or accentuated as dose increases while varying with biochemical events underlying the observed effects. Therefore, it is likely that isoflavone effects on adipokine secretion and adiponectin regulation of Leydig cells will depend on both the length of exposure and dosage.

The high concentrations of adiponectin in the serum (micromolar levels) have brought its physiological relevance into question (Stefan and Stumvoli, 2002). However, serum adiponectin levels are increased under conditions of persistent negative energy balance, e.g., during starvation, and it is possible that elevated serum adiponectin contributes to inhibition of testicular steroidogenesis under these conditions (Caminos et al., 2008). On the other hand, coexpression of adiponectin and adipoR2 in Leydig cells suggests the occurrence of a distinct autocrine pathway for adiponectin regulation of androgen secretion. In particular, we observed that T production was decreased in the 1000 ppm diet group, whereas adiponectin and adipoR2 protein was affected by developmental exposure to all levels of isoflavones. These results raise the possibility that disruption of the autocrine loop in Leydig cells, affecting adiponectin and adipoR2, have consequences for testis function. However, the physiological relevance of autocrine regulation of Leydig cells by adiponectin warrants further investigation.

Interestingly, adiponectin and insulin appear to cause inhibition of androgen biosynthesis acting through different mechanisms: adiponectin suppresses StAR protein, whereas insulin decreases the rate of StAR phosphorylation. Because adiponectin has insulin-sensitizing properties, it is not likely that both adiponectin and insulin will be present at high levels in blood at the same time. Nevertheless, the differential effects of adiponectin and insulin in Leydig cells suggest that metabolic factors affect testicular cells through multiple mechanisms. Insulin-mediated decreases in StAR phosphorylation also indicate that insulin action affecting the testis is mediated both centrally, i.e., through GnRH release from the hypothalamus (Koch et al., 2008), and peripherally, i.e., in Leydig cells (this study).

In conclusion, the present results imply that metabolic syndromes affecting adipokine secretion may affect the endocrine function of the testis. In addition, the data demonstrated that adiponectin can act directly in Leydig cells and possibly mediate the effects of energy balance on male reproduction. Additional studies are required to investigate these possibilities.

Conflict of interest

No financial or other conflict of interest to disclose.

Acknowledgements

This study was supported in part by the Animal Health and Disease Research, Boshell Diabetes Research and the Biogrant Programs at Auburn University. Data were presented in a preliminary form at the 92nd Annual Meeting of the Endocrine Society, June 19–22, 2010, San Diego, CA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2011.11.027.

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