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Abbreviations:

AUC Area under the curve

BPA Bisphenol-A

EDCs Endocrine disrupting chemicals

IPGTT Intraperitonoal Glucose tolerance test

IPITT Intraperitoneal Insulin Tolerance Test

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ABSTRACT

Background: Bisphenol-A (BPA) is a widespread endocrine disrupting chemicals (EDCs) used as the base compound in the manufacture of polycarbonate plastics. In humans, epidemiological evidence associated Bisphenol-A exposure in adults with higher risk of type-2 diabetes and heart disease.

Objective: Here we studied the action of environmentally relevant doses of BPA on glucose metabolism during pregnancy and the impact of BPA exposure on these females later in life. We investigated the consequences of *in utero* exposure to BPA on metabolic parameters and pancreatic function in offspring.

Methods: Pregnant mice were treated with either vehicle or BPA (10 or 100µg/kg/day) during days 9-16 of gestation. Glucose metabolism experiments were performed on pregnant mice and their offspring.

Results: Exposure to BPA aggravated the insulin resistance produced during pregnancy and was associated with decreased glucose tolerance and increased plasma insulin, triglyceride and leptin concentrations relative to controls. Insulin-stimulated Akt phosphorylation was reduced in skeletal muscle and liver of BPA-treated pregnant mice relative to controls. BPA exposure during gestation had long-term consequences for mothers: four months post-partum, mice weighed more than untreated females, and had higher plasma insulin, leptin, triglyceride and glycerol levels and greater insulin resistance. At six months of age *in utero* exposed male offspring had reduced glucose tolerance, increased insulin resistance, and altered blood parameters compared with offspring of untreated mothers. The islets of Langerhans from male offspring presented altered Ca^{2+} signaling and insulin secretion. BrdU incorporation into insulin-producing cells was reduced in the male progeny yet beta-cell mass was unchanged.

Conclusions: Our findings suggest that BPA may contribute to metabolic disorders relevant to glucose homeostasis, and that BPA may be a risk factor for diabetes.

INTRODUCTION

Research of the last decade has revealed that conditions experienced during early development play an important role in determining the long-term health of individuals. Alterations in development due to impaired maternal metabolism can lead to the permanent programming of physiological systems. Gestation generates a state of increased metabolic demand to ensure a balance between maternal and fetal requirements. To meet the demands of pregnancy, a coordinated series of maternal adaptations occur including changes of metabolic processing within different tissues and changes in nutrient partitioning that ensure proper growth of the fetus (Ryan 2003). The most profound of these adaptations occurs with glucose metabolism since glucose is the primary nutrient for fetal growth and milk synthesis. Thus, glucose production increases during late pregnancy and early lactation and concurrently, glucose uptake by muscle and adipose tissue progressively declines. This insulin-resistant state ensures that an adequate supply of glucose is shunted to the growing fetus (Barbour et al. 2007; King 2006). Despite this condition, serum glucose concentrations are maintained within the physiological range since the maternal endocrine pancreas adapts by increasing insulin secretion. If this adjustment fails, gestational diabetes ensues (Kuhl 1998). Experimental and epidemiological data suggest that gestational diabetes may have long-term consequences for both baby and mother, including a predisposition to obesity, metabolic syndrome, and diabetes later in life (Boloker et al. 2002; Reece et al. 2009).

In addition to insulin, gestation requires significant changes to other hormones. The rise of maternal serum levels of prolactin, placental lactogens, progesterone and estradiol in late pregnancy is related, at least in part, with the development of insulin resistance (Gonzalez et al. 2003; Nadal et al. 2009a). Among these hormonal adaptations, those related with estradiol (E2) are key. In addition to its role in the physiology of reproduction, E2 has been

proposed to mediate maternal adaptation to the enhanced demand for insulin because it enhances insulin biosynthesis as well as glucose-stimulated insulin secretion (Nadal et al. 2009a). Although physiological levels of estradiol are involved in maintaining normal insulin sensitivity (Liu and Mauvais-Jarvis 2010; Louet et al. 2004), E2 outside of the physiological range may have adverse effects on glucose homeostasis (Livingstone and Collison 2002; Nadal et al. 2009b).

Recently, environmental estrogens such as BPA have become public health concerns because of experimental evidence indicating deleterious effects on energy balance and glucose homeostasis in animal models (Newbold et al. 2008; Alonso-Magdalena et al 2006, 2008; Nadal et al. 2009b). In humans, BPA has been associated epidemiologically with type-2 diabetes and heart disease (Lang et al, 2008). Although first discovered as a synthetic estrogen (Dodds and Lawson 1936), BPA is currently used as the base compound in the manufacture of polycarbonate plastic and the resin lining of food beverage cans and drinking water bottles and containers (vom Saal et al, 2007). Importantly, BPA has been shown to leach from polycarbonate containers and consequently, BPA has been widely detected in humans. Indeed, the potential risk for BPA exposure is emphasized by the finding that BPA was found in 98% of the urine samples from residents of USA (Calafat et al. 2008). Its concentration in human serum ranges from 0.2 to 1.6 ng/ml (0.88-7.0 nM). In addition, it is has been detected in amniotic fluid, neonatal blood, placenta, cord blood and human breast milk, demonstrating the potential of this compound to pass from mother to the fetus (Vandenberg et al, 2007). A large number of *in vivo* and *in vitro* studies have reported adverse effects associated with BPA, and interestingly, many were caused by concentrations below the predicted "safe" reference dose of 50 μg/kg/day established by the U.S.-EPA (vom Saal et al. 2007). In the present study, we chose low doses of BPA based on the U.S.-EPA criterion for low-dose effects of EDCs. Levels below the current

lowest observed effect level (LOAEL) of 50 mg/kg/day were considered low dose for *in vivo* studies (Environmental Protection Agency (EPA); Wetherill et al. 2007). Although the initial concerns about BPA were related to reproductive parameters and its carcinogenic potential, few studies have examined the consequences of BPA exposure during pregnancy on the mother, and no study has assessed the potential risk for developing diabetes, despite the fact that gestational diabetes is a major potential complication of pregnancy with adverse consequences for both mothers and newborns.

In the present work, we demonstrate that low concentrations of BPA have deleterious long-term effects on glucose metabolism in mice during pregnancy and post-partum as well as in their adult offspring. Our results suggest that BPA exposure could contribute to the development of gestational diabetes, obesity, and a pre-diabetic state later in life. Notably, in utero exposure to BPA was associated with decreased glucose tolerance and increased insulin resistance in male offspring at six months of age compared with controls, consistent with an effect of BPA on fetal programming that could predispose adult mice to type-2 diabetes and metabolic disorders.

RESEARCH DESIGN AND METHODS

Animals and treatment

Pregnant mice OF-1 were purchased from Charles River and individually housed under standard housing conditions. Mice were maintained on chow from Harlan Laboratories (Ref 2014) which does not contain alfalfa or soybean meal. The macronutrients composition is as follow: crude protein 14.3%, fat 4%, carbohydrate 48%, crude fiber 4.1%, neutral detergent fiber 18%, ash 4.7%, energy density 2.9 (12.1) kcal/g (kJ/g), calories from protein 20%, calories from fat 13%, calories from carbohydrate 67%.

Experimental procedures were reviewed and approved by an institutional committee for animal care and use. Animals were treated humanely and with regard for alleviation of suffering.

BPA or vehicle (saline) was dissolved in tocopherol-stripped corn oil and administered subcutaneously on days 9 through 16 of gestation. The daily dose used was 10 or 100 μg/kg. We observed no significant difference in litter size between control and BPA-treated mice. Pups of the same treatment group were pooled together, and then placed in equal numbers with foster mothers of the same treatment group. Pups were separated by sex for placement with foster mothers.

Glucose and insulin tolerance test

For glucose tolerance tests, animals were fasted overnight for 12 hr, and blood samples were obtained from the tail vein. Animals were then injected intraperitoneally with 2 g/kg body weight of glucose, and blood samples were taken at the indicated intervals. For insulin tolerance tests, fed animals were used. Animals were injected intra-peritoneally with 0.75 or 1.25 IU/kg body weight of soluble insulin. Blood glucose was measured in each sample using an Accu-check compact glucometer (Roche, Madrid, Spain).

Plasma analysis

To measure plasma metabolites, mice were anesthetized with 50 mg/kg body weight sodium pentobarbital. Blood was obtained by cardiac puncture. Insulin and leptin were measured by enzyme-linked immunoabsorbent assay (Mercodia, Uppsala,Sweden and Crystal Chem, Downers Grove IL, USA, respectively) triglycerides and glycerol (GTO-Trinder Triglycerides assay, Sigma, Madrid, Spain).

Insulin secretion measurement

Pancreatic islets of Langerhans from F1 6 months old mice were isolated by collagenase (Sigma) digestion as previously described (Alonso-Magdalena et al, 2006). Islets were collected with a micropipette one by one. Islets were used immediately after isolation. Islets were washed twice with a buffer solution containing 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 3 mM D-glucose, final pH = 7.35. Groups of 5 islets were then incubated in 1 mL of this buffer in the presence of 3, 7, and 16 mM glucose. After 1 hr, the medium was collected, and insulin was measured in duplicate samples by radioimmunoassay using a Coat-a-Count kit (DPC, Los Angeles, CA, USA). Protein concentration was measured by the Bradford dye method (Bradford, 1976).

Recording $[Ca^{2+}]_i$

Isolated islets of Langerhans were loaded with 5 μM fura 2-AM for at least 1 h at room temperature. Calcium records in the whole islet of Langerhans were obtained by imaging intracellular calcium under an inverted epifluorescence microscope (Axiovert 200; Carl Zeiss GmbH, Jena, Germany). Images were acquired every 2 s with an extended Hamamatsu Digital Camera C4742-95 (Hamamatsu Photonics, Barcelona, Spain) using a dual-filter wheel equipped with 340 and 380 nm, 10-nm bandpass filters. Data were acquired using Aquacosmos software from Hamamatsu (Hamamatsu Photonics, Barcelona, Spain). Fluorescence changes are expressed as the ratio (R) of fluorescence at 340 and 380 nm (F_{340}/F_{380}). Results were plotted and analyzed with use of commercially available software (Sigmaplot, SPSS Inc., Chicago, IL).

Western Blot

Insulin signaling experiments were conducted for western blot analysis. Briefly, pregnant mice were fasted 4 hours and received a single intraperitoneal injection of 0.6 units/kg insulin and tissues were harvested 10 min later. Gastrocnemius muscles and liver were homogenized in ice-cold buffer (10% glycerol, 20mM sodium pyrophosphate, 150 mM NaCl, 50 mM Hepes (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2 mM sodium orthovanadate, 3 mM benzamidine (pH 7.4) for 20 sec. Homogenates were rotated end-over-end for 1h at 4˚C and subjected to centrifugation (14000 g for 10 min) at 4˚C. Protein content in lysates was measured by the Bradford method. Muscle and liver lysates were adjusted to equal protein concentration, boiled in Laemmli-buffer, and loaded on 7.5% gels. Membranes were blocked in TBST buffer (10 mM Tris-base, 150 mM NaCl, 0.25% Tween 20) containing 5% low fat milk protein for 2h at room temperature. Membranes were then incubated with primary antibodies overnight at 4˚C, washed with TBST buffer followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Richmond, CA) for 1h at room temperature.

Akt phosphorylation was determined by using an antibody against phospho-Akt (Threonine ^{308}) (1:1000; Cell signaling). An anti-Akt antibody was used to confirm equal loading and normalize samples. Protein bands were revealed by using the ECL Chemiluminiscence Reagents kit (Amersham Biosciences, Barcelona, Spain). Intensity of the bands was quantified using Scion image software (Frederick, MD USA).

Assessment of pancreatic β-cell area

Pancreata were removed at the time of sacrifice (F1, 6 months old mice) and fixed overnight in 4% paraformaldehyde. Subsequently, pancreatic tissue was embedded in paraffin and sections of 5 µm were prepared. Following re-hydration and permeabilization (1 % Triton X-100), sections were incubated with anti-insulin (Sigma, Madrid, Spain) and anti-glucagon (Sigma, Madrid, Spain) antibodies overnight at 4°C. Detection was performed with rhodamine and fluorescein conjugated secondary antibodies (Jackson Immunoresearch, Suffolk, UK). For quantification of ß-cell area, sections were viewed at a magnification of 10x. The islet cross-sectional area and total pancreatic area were measured using ImageJ analysis program. At least 3 sections, separated by 200 µm were measured per animal. For quantification of the number of islets per area, only islets with more than 5 positive-stained cells were scored.

Analysis of BrdU incorporation

Mice were given intraperitoneal injections of BrdU (100 µg/g) four hours before sacrifice. Mice were the same used for assessment of pancreatic β-cell area. Pancreas tissue was fixed and processed as described above. After dehydration, sections were heated to 100 C° in the presence of citrate buffer (10mM) for 5 min. Slides were then blocked by incubating for 30 min in 0.1% BSA and 5% normal goat serum in PBS/0.2% TX-100. Samples were then incubated with antibodies for insulin (1:200, rabbit polyclonal, Santa Cruz, Madrid, Spain) and BrdU (1:200, monoclonal, DAKO, Barcelona, Spain) overnight at 4ºC. After incubation with secondary antibodies, sections were mounted using Fluorsave (Calbiochem, Madrid, Spain). Images were acquired from double-stained sections. BrdU+ nuclei were scored only in cells which

were also positive for insulin. Quantification was done on at least 3 sections, separated by 200 μm, from each animal.

Statistical analysis

The SigmaStat 3.1 software package (Systat Software, Inc., Chicago, IL, USA), was used for all statistical analyses. One-way ANOVA followed by Bonferroni post hoc test was used to assess differences between treatment groups for each exposure paradigm. Results in Figure 1C and D were analyzed by Two-way ANOVA followed by Fisher LSD test. When data did not pass parametric test, we used ANOVA on RANKS followed by Dunn´s test (stated in figure legends). Results were considered significant at $p<0.05$. All results are expressed as mean±S.E.M.

RESULTS

BPA exposure during pregnancy: consequences for maternal glucose homeostasis

To examine the effects of BPA on maternal glucose metabolism, we treated pregnant mice with either vehicle or BPA at doses of 10 or 100 μg/kg/day from day 9 to 16 of gestation. Subsequently, we measured glucose and insulin sensitivity as well as plasma metabolites on days 16-18 of gestation. Across the groups, animals were matched for gestation day to minimize potential differences in the background levels of maternal hormones during the last phase of pregnancy. Vehicle-treated pregnant mice are referred to as control (F0-C), while pregnant mice which received BPA at 10 or 100μg/kg/day are designated as F0-BPA10 and F0-BPA100, respectively.

Results of the intraperitoneal glucose tolerance test (IPGTT) revealed that F0-BPA10 displayed glucose intolerance compared to F0-C (Figure 1A). The F0-BPA100 group displayed a tendency to glucose intolerance, yet the area under the curve (AUC), an index of glucose tolerance, was not significantly different when compared to vehicle-treated animals (Figure 1A inset). Intraperitoneal insulin tolerance tests (IPITT) were performed to assess insulin sensitivity. In both F0-C and F0-BPA10, insulin caused only a modest decrease in serum glucose levels, reflecting the physiological insulin resistance which develops during late pregnancy. However, we observed a tendency to increased insulin sensitivity in F0-BPA100, although this did not reach statistical significance (Figure 1B).

Since BPA treatment during pregnancy led to altered glucose homeostasis, particularly in the F0-BPA10 group, we next studied signalling pathways in liver and skeletal muscle of pregnant mice which had received intraperintoneal injections of insulin. In liver from F0-C mice, insulin increased the phosphorylation of Akt as compared to saline treatment (Figure 1C). In contrast, insulin stimulation actually decreased Akt phosphorylation in liver of F0-

BPA-10 mice, suggesting that BPA treatment impairs hepatic insulin signalling. In gastrocnemius muscle (Figure 1D), insulin increased Akt phosphorylation in the F0-C group whereas this response was completely blunted in the F0-BPA10 group, consistent with severe insulin resistance. These results demonstrate that the lowest dose of BPA had considerable effects on glucose homeostasis and enhanced insulin resistance in both liver and muscle from pregnant mice. Consistent with this, hyperinsulinemia was detected in both F0-BPA10 and F0-BPA100 relative to F0-C (Table 1). When other blood parameters were analyzed, F0-BPA100 exhibited higher levels of plasma triglycerides and glycerol as well as increased plasma leptin compared to F0-C (Table 1).

BPA exposure during pregnancy: consequences for mothers later in life

To study whether BPA exposure during pregnancy is detrimental to glucose metabolism in females after parturition, we treated pregnant mice as described above and monitored metabolic parameters in these animals for several months post-partum. Three months after delivery, animals that had been treated with BPA showed an increase of body weight which was more pronounced in the F0-BPA100 group (Supplemental Material Figure 1A). At this point of evaluation, no statistically significant differences in insulin sensitivity were observed in F0-BPA100 (Supplemental Material Figure 1B).

Four months after delivery, the increased body weight associated with BPA exposure persisted (Figure 2A), without differences in food intake measured during 6 days in mice 4 months after delivery (Supplemental Material Figure 1C). When IPITT was performed, the glucose-lowering effects of insulin were attenuated in F0-BPA100 as compared with F0-C (Figure 2B). Consistent with this, we observed that glucose tolerance was clearly altered in F0-BPA100 (Figure 2C). Accordingly, fed plasma insulin levels were 2.2 times higher in

F0-BPA100 than in control mice (Table 2), while glucose levels did not differ. These observations reinforced the idea that F0-BPA100 animals develop insulin resistance. Moreover, the higher plasma insulin levels were accompanied by increased plasma leptin, triglyceride and glycerol levels relative to controls (Table 2). However, in the case of F0-BPA10, no differences were observed in IPITT and IPGTT (Fig 2B, C), and only triglyceride levels were statistically different from control animals (Table 2). Nevertheless, it should be noted that plasma insulin, glycerol and leptin levels were also increased, although the increases were not statistically significant (Table 2). Collectively, these results suggest that exposure to a dose of BPA 100µg/kg/day during pregnancy has persistent metabolic consequences which, with time, lead to insulin resistance and dysregulated nutrient metabolism.

BPA exposure during pregnancy: fetal programming of type-2 diabetes in adult offspring

To test whether BPA exposure *in utero* predisposes for future development of metabolic abnormalities, we studied three groups of animals: offspring from vehicle-treated mothers (F1-C), offspring from BPA 10µg/kg/day treated mothers (F1-BPA10) and offspring from BPA 100µg/kg/day treated mothers (F1-BPA100). Note that these offspring received no direct treatment with BPA; their mothers were treated during days 9-16 of pregnancy.

F1-BPA10 mice were 3% heavier than F1-C mice at birth and 7% heavier at weaning (22 days postpartum). In contrast, body weight in the F1-BPA100 group was 4.5% lower than F1-C controls at birth, and the relative difference persisted until weaning (Figure 3A and B). In male offspring body weight was comparable among experimental groups from weaning through 6 months of age (Figure 3C). In female offspring body weight was also

comparable among groups in the post-natal period, but at 3 months of age F1-BPA10 and F1-BPA100 mice weighed 3% and 2.5 % more than F1-C controls, respectively (Figure 3D).

At 3 months of age, we performed IPITT to evaluate insulin sensitivity of the offspring. Insulin sensitivity was slightly decreased in F1-BPA10 male offspring as compared to control but not in F1-BPA100. Nevertheless, differences were not statistically significant (Supplemental Material Figure 2A). No differences were detected in female offspring (Supplemental Material Figure 2B).

At 6 months of age, insulin sensitivity was clearly impaired in F1-BPA10 males as shown in figure 4A, yet insulin sensitivity was normal in F1-BPA100. Glucose tolerance was altered in both F1-BPA10 and F1-BPA100: the AUC was significantly increased in F1-BPA10 and non-significantly increased in F1-BPA100 mice relative to F1-C controls (Figure 4B inset). In 6 months female offspring, no differences were found in insulin sensitivity (Supplemental Material Figure 2C) and glucose tolerance (Supplemental material Figure 2D). Therefore, subsequent studies were performed only with male animals.

Serum insulin and glycerol levels were higher in F1-BPA10 compared with F1-C controls, but leptin levels were comparable (Table 3). Interestingly, in F1-BPA100, only glycerol levels were significantly increased relative to controls. To relate these observations with alterations in the endocrine pancreas, we studied glucose-stimulated insulin secretion 15 min after a glucose load in vivo and ex vivo. Glucose-stimulated insulin secretion was doubled in F1-BPA10 mice relative to controls, but was comparable in F1-BPA100 mice (Figure 5A). Following ex vivo exposure to high glucose concentrations (16 mM), islets from F1-BPA10 mice secreted 1.53 times more insulin than

islets from control mice, whereas islets from F1-BPA100 mice secreted slightly less insulin than controls, though the difference was not statistically significant (Figure 5B). Enhanced insulin secretion in F1-BPA10 mice was accompanied by a greater increase in global intracellular Ca^{2+} entry following glucose stimulation. However, in the F1-BPA100 group, the increase in global Ca^{2+} entry was slightly less than the response observed in controls (not statistically significant) (Fig 5C and D). We next studied whether pancreatic β-cell mass was altered in BPA-treated mice. Although there was no change in β-cell area among the groups (Figure 5E), BrdU incorporation into β-cells, a marker of proliferation, was markedly reduced in both F1-BPA10 and F1-BPA100 mice relative to F1-C (Figure 5F).

DISCUSSION

In the present study, we have demonstrated that exposure to low doses of BPA during critical periods of life has adverse effects on glucose homeostasis and insulin sensitivity. When pregnant mice were treated with BPA on days 9-16 of gestation, they developed glucose intolerance and elevated levels plasma insulin, triglycerides, glycerol and leptin compared with control pregnant mice. Interestingly, this brief exposure to BPA had long-term metabolic consequences for both the mother and male offspring.

BPA exposure in mothers: consequences for glucose homeostasis during pregnancy

Maternal adaptation to pregnancy is mediated mainly by placental hormones, such as prolactin, placental lactogens and steroid hormones, including estrogens (Nadal et al. 2009a). Estrogen signalling is emerging as a key pathway in glucose and lipid metabolism and may play an important role in the insulin resistance which occurs during pregnancy (Gonzalez et al. 2003). In the pancreas, estrogen receptors are important in the regulation of insulin biosynthesis and release, a process that appears to counteract the increased insulin resistance associated with pregnancy (Nadal et al, 2009a). Bisphenol-A is a xenoestrogen and its estrogenic effect alters pancreatic β-cell function and induces glucose intolerance and insulin resistance in male mice (Alonso-Magdalena et al 2006). Moreover, its effect in β-cells is direct and occurs via the estrogen receptor α (ERα) (Alonso-Magdalena et al, 2008). In addition, BPA binding to extranuclear ERα alters prolactin secretion (Wozniak et al, 2005). Therefore, alterations of estrogen signalling by BPA treatment during pregnancy would be expected to produce deleterious effects on the adaptation of the endocrine pancreas, pituitary, and other peripheral tissues.

Results of the present study indicate that, compared with controls, mice treated with BPA 10μg/kg/day during pregnancy had impaired glucose tolerance, slightly increased fasting

plasma insulin levels (1.38 times higher than in controls), and reduced insulin sensitivity in skeletal muscle and liver (as evidenced by the inability of insulin to promote phosphorylation of Akt at Thr 308 in these tissues) (Alessi et al. 1996). Thus, the reduction in glucose tolerance observed in F0-BPA10 mice compared with controls may reflect an inability to increase insulin levels enough to compensate for the observed increase in insulin resistance In contrast, higher fasting plasma insulin levels in mothers treated with BPA 100μg/kg/day (F0-BPA100) (double the mean concentration in controls) may have partially compensated for the relative increase in insulin resistance that was also observed in F0-BPA100 mice, and may explain why F0-BPA100 mice tolerated a glucose challenge better than F0-BPA10 mice. In addition, fasting plasma leptin levels were 2.1 times higher in F0-BPA100 mice than controls. A major function of leptin is regulation of glucose metabolism and insulin sensitivity in muscle, liver and endocrine pancreas (Tudurí et al. 2009; Wang et al. 2010). Leptin levels are increased during pregnancy and leptin receptors are present in maternal tissue, placenta and fetal tissue (Sagawa et al. 2002). Therefore, increased levels of leptin may affect energy metabolism in mothers and fetal growth. It will be of interest to determine whether BPA directly regulates leptin release from adipocytes, as is the case of adiponectin secretion (Hugo et al, 2008), or whether the observed hyperleptinaemia is a consequence of the altered metabolic state of these animals.

BPA treatment during pregnancy affects mother's glucose metabolism later in life

Experimental and observational data suggest that gestational diabetes may increase a mother's risk for obesity and type-2 diabetes later in life (Boloker et al. 2002; Reece et al. 2009). Mothers treated with 10μg/kg/day of BPA during pregnancy weighed more (not significant) and had higher plasma triglyceride levels 4 months after delivery than controls.

It is important to note that this BPA dose is five-times lower than the dose considered completely safe during a lifetime by the US-EPA. The effects observed with 100 μg/kg/day of BPA, only twice the level considered safe during lifetime by US-EPA, were even more striking. With the higher dose, pregnant mice demonstrated increased insulin resistance and decreased glucose tolerance, and significantly higher fasting plasma insulin, leptin, triglyceride and glycerol levels than controls. In humans, these metabolic alterations are associated with an increased risk of type-2 diabetes and cardiovascular disease (Kahn et al. 2006). Interestingly, increased insulin resistance in F0-BPA100 mice was not evident 3 months after delivery, but was observed 4 months after delivery, which suggests that insulin resistance resolved after parturition but was triggered several months later by a yet unknown mechanism.

Effects of EDCs, including those of BPA, are usually classified as “activational” and “organizational”. The first term refers to effects resulting from adult exposure, and they are generally considered to be reversible. The second term refers to effects resulting from perinatal exposure and it is well accepted that they can persist even in the absence of subsequent re-exposure (Richter et al. 2007). In the present work, we described an effect of BPA in adults that appeared during gestational exposure, disappeared after labour and reappeared later in life. Thus, these results challenge the long-standing assumption that effects of exposure to endocrine disruptors during adulthood are reversible after exposure ceases. The mechanistic basis of these alterations is not yet known, although it is plausible that epigenetic modifications of maternal tissues may influence metabolism later in life and perhaps, during subsequent pregnancies.

BPA treatment during pregnancy: implications for offspring metabolism

It is well known that conditions experienced during the intrauterine period can have lifelong effects on health, the so-called "fetal plasticity" theory (Barker 1998; Gilbert and Epel 2009). Experimental and epidemiological studies in rodents and humans suggest that high/low birth weight is a risk factor of type 2 diabetes later in life (Gilbert and Epel 2009). It has been demonstrated that mice with low birth weight from malnourished mothers present glucose intolerance and β-cell failure during adulthood (Jimenez-Chillaron et al. 2005). Exposure to EDCs, such as diesthylstylbestrol (DES) *in utero* provokes various diseases in offspring during adulthood (McLachlan et al. 2001). Although information related to EDC-induced metabolic syndrome is sparse, it is known that uterine exposure to DES induced high birth weight and obesity in mice adult offspring (Newbold et al. 2008, 2009). Moreover, *in utero* exposure to low doses (25 µg/kg/day) of BPA in rats is associated with high birth weight (Rubin and Soto 2009).

Our results demonstrate that exposure during pregnancy to 10 µg/kg/day BPA was associated with higher birth weight. This may have reflected effects on the metabolic state of the mothers (F0-BPA10), mainly reduced glucose tolerance and increased plasma insulin levels, which are factors that may influence fetal growth (Reece 2009). Offspring (F1-BPA10) also demonstrated reduced glucose tolerance, increased insulin resistance, and higher levels of plasma insulin and glycerol (an indicator of high free fatty acids). In addition, beta cells from F1-BPA10 offspring were more sensitive to extracellular glucose, both in vivo and ex vivo. This enhanced secretion of insulin may represent a homeostatic mechanism to compensate for an increase in peripheral insulin resistance. However, the incorporation of BrdU, which was used to assess beta cell proliferation, was significantly reduced in both groups of BPA-exposed offspring as compared with F1-C. This seeming contradiction between preserved beta cell mass and impaired BrdU incorporation might be explained by the effects of BPA on beta cell turnover. Although adult beta cells expand

during adulthood to match peripheral demands for insulin, recent studies have demonstrated that in fact beta cells of aged mice reside in a mostly quiescent state and display a very low rate of turnover (Teta et al. 2005, Teta et al. 2007). Thus, it is plausible that *in utero* exposure to BPA alters cell-cycle machinery without provoking apoptosis of beta cells and given that insulin secretion is enhanced, diabetes can be avoided in these animals until the increased demand for insulin reaches a point where expansion of beta cell is also required to compensate for insulin resistance.

Notably, the metabolic phenotype of F1-BPA100 is quite different from the low dose group. F1-BPA100 had lower birth weights than F1-C. Since mothers (F0-BPA100) presented milder glucose intolerance and higher levels of leptin than F0-BPA10, this may influence birth weight and therefore, glucose homeostasis of offspring. In addition, F1-BPA100 presented milder glucose intolerance than F1-BPA10, normal insulin sensitivity, and plasma insulin levels that were not statistically different from controls. When pancreatic beta cell function was analyzed in vivo we found that the insulin response to an intraperitoneal injection of glucose was reduced in F1-BPA100 mice relative to controls. Experiments ex *vivo* with isolated islets indicate that glucose–induced Ca^{2+} signals and insulin release were only slightly diminished with respect to control. Therefore, F1-BPA100 animals presented glucose intolerance likely due to liver insulin resistance rather than to alterations in the β-cell stimulus-secretion coupling.

It must be noted that only male offspring had altered glucose tolerance and insulin resistance since age-matched F1-BPA females displayed normal metabolic parameters. Females are protected against insulin resistance more than males, in part because the presence of estrogens, which have been demonstrated that, within the physiological range, protect against diabetes in mice (Louet and Mauvais-Jarvis, 2004; Liu and Mauvais-Jarvis, 2010). Moreover, at 3 months of age, neither males nor females presented insulin resistance

nor glucose intolerance. Ryan et al. 2010 have described similar results in their study of glucose tolerance until 15 weeks of age, concluding that perinatal exposure to BPA (0.25μg/kg/day) did not affect glucose homeostasis in adult mice. However, in the present work, although the BPA treatment protocol was different, metabolic alterations did not appear until later in adulthood, at 6 months of age.

The "developmental" or "fetal" origin of adult disease hypothesis states that environmental factors act early in life to program the risks of developing chronic diseases in adult life. In our model of study, metabolic effects observed in offspring mice exposed to BPA may be due to two factors: abnormal hormonal environment and altered glucose metabolism. Whether the effects we have observed in the offspring are due to a direct effect of BPA on the fetus or because the fetus is exposed to an altered maternal metabolism, or the combination of both factors, remains unknown. Nevertheless, the latter is the most plausible situation, since BPA crosses the placenta and glucose tolerance, insulin and leptin signaling during gestation are important for fetal growth. Indeed, maternal metabolism is important because the impaired glucose tolerance and blood parameters observed in mothers treated with 10 or 100 μg/Kg/day may explain, at least in part, the different metabolic abnormalities displayed subsequently in offspring F1-BPA10 compared to F1-BPA100.

In any case, the results of this study suggest that the endocrine disruptor bisphenol-A should be evaluated as a possible risk factor for gestational diabetes, type-2 diabetes, and even cardiovascular disease associated with metabolic syndrome. Moreover, our findings in mice suggest that fetal exposure to BPA may predispose males to type-2 diabetes during adulthood.

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End point	Vehicle	F0-BPA10	F0-BPA100
Insulin (µg/L)	0.82±0.13	1.13±0.15*	1.99±0.37**
TG (mg/mL)	1.07±0.15	1.71±0.25	2.04±0.36*
Glycerol (mg/mL)	0.33±0.04	0.55±0.07	0.87±0.15*
Leptin (mg/mL)	1.2±0.15	1.35±0.11	2.52±0.31*

Table 1. Plasma hormone and metabolite levels after 4 hours fasting in mice at day 18 of pregnancy

*$p<0.05$, **$p<0.005$, One Way ANOVA followed by Bonferroni compared to vehicle, n=8-13 mice. Parameters were measured after 4 hours fasting.

End point	Vehicle	F0-BPA10	F0-BPA100
Insulin (μg/L)	0.94±0.20	1.61±0.17	2.08±0.39*
TG (mg/mL)	0.44±0.07	1.22±0.29[a]*	1.97±0.30[a]*
Glycerol (mg/mL)	0.27±0.03	0.66±0.17	0.94±0.19*
Leptin (mg/mL)	2.70±0.59	5.33±1.12	6.05±1.06*

Table 2. Plasma hormone and metabolite levels after 4 hour fasting in BPA-treated mice after delivery.

$p<0.05$, One Way ANOVA followed by Bonferroni; [a]$p<0.05$, One Way ANOVA followed by Holm-Sidak, n=6 mice/group. Parameters were measured after 4 hours fasting.

End point	Vehicle	F1-BPA10	F1-BPA100
Insulin (µg/L)	0.85±0.13	1.73±0.25*	1.40±0.18
TG (mg/mL)	0.84±0.16	1.41±0.03	1.25±0.09
Glycerol (mg/mL)	0.18±0.02	0.27±0.02[a]*	0.24±0.02[a]*
Leptin (mg/mL)	1.69±0.42	1.78±0.47	1.85±0.44

Table 3. Plasma hormone and metabolite levels after 4 hours fasting in male offspring at 6 months of age

$p<0.05$, One Way ANOVA followed by Bonferroni; [a]$p<0.05$, One Way ANOVA followed by Holm- Sidak, compared with Vehicle, 6-13 mice/group. Parameters were measured after 4 hours fasting.

FIGURE LEGENDS

Figure 1. BPA exposure during pregnancy alters blood glucose homeostasis.

(A) An intraperitoneal glucose tolerance test (IPGTT) was performed in pregnant mice previously treated with vehicle (n=11), or 10 or 100 µg/kg/day BPA (n=8) on days 9 through 16 of gestation. Inset: mean total area under the curve in response to glucose load in the vehicle and BPA pregnant-treated mice. (B) An intraperitoneal insulin tolerance test (IPITT) in fed pregnant mice treated with vehicle (n=12), BPA 10 (n=9) or BPA100 µg/kg/day (n=7). (C) Western blot analysis of insulin-stimulated Akt phosphorylation (Thr308) in liver (D) Western blot analysis of insulin-stimulated Akt phosphorylation (Thr308) in gastrocnemius muscle. Tissue for these experiments was collected 15 min after intraperitoneal injection of 0.6 units/kg insulin or saline in vehicle-treated mice (n=4) and BPA10µg/Kg/day treated mice (n=6). *$p < 0.05$. Data are expressed as mean±S.E.M.

Figure 2. Follow-up of glucose and insulin sensitivity in BPA- treated females after delivery.

(A) Mean body weights of mothers treated with vehicle, BPA10 or 100 µg/Kg/day four months post-delivery. (B) An intraperitoneal insulin tolerance test (IPITT) performed in vehicle, BPA 10 and 100 µg/Kg/day in mothers 4 months after parturition. (C) An intraperitoneal glucose tolerance test (IPGTT) in the same mothers as B. Inset represents the area under the curve (AUC). n=6, *$p < 0.05$. Data are expressed as mean±S.E.M.

Figure 3. Body weight of offspring from mice exposed to BPA during gestation.

(A) Mean body weights from birth to weaning of offspring born to mothers exposed to BPA (10 or 100 µg/kg/day) or vehicle during days 9 to 16 of gestation. (B) Same as in A but expressed as percentage. (C) Mean body weight of male offspring from weaning to adulthood (22 through 180 days of age). (D) Mean body weight of female offspring from weaning to adulthood (22 through 180 days of age). n=25-60 animals/group, $^*p<0.05$. Some SEMs are not visible in the graph because of their low values. Data are expressed as mean±S.E.M.

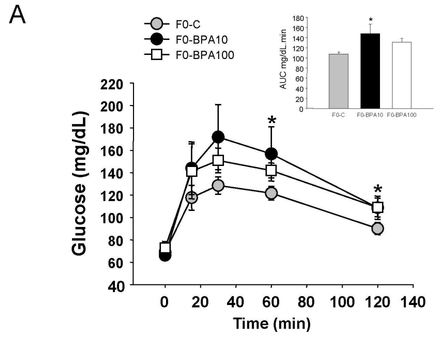
Figure 4. Altered blood glucose homeostasis in male offspring of mice exposed to BPA or vehicle.

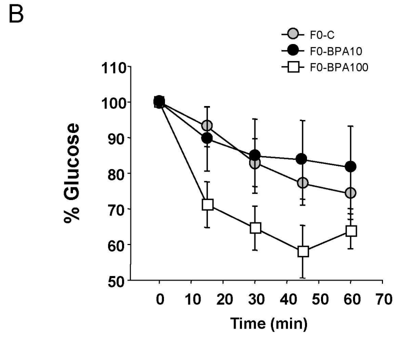
(A) Insulin sensitivity (IPITT) of male offspring from mice treated with BPA (10 or100 µg/Kg) or vehicle performed at 6 months of age. (B) Glucose tolerance test (IPGTT) performed in the same group of animals. Inset represents mean total area under the glucose curve. n=8 $^*p< 0.05$. Data are expressed as mean±S.E.M.

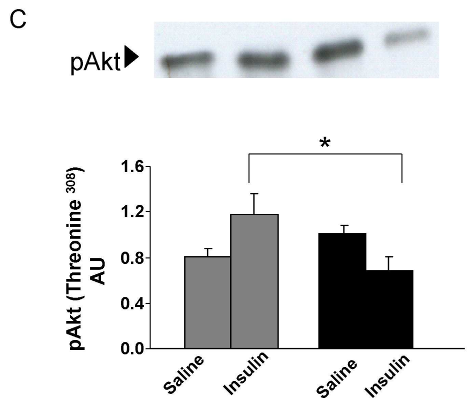
Figure 5. Pancreatic islet function in male offspring of mice exposed to BPA or vehicle.

(A) In vivo plasma insulin levels 15 minutes after a glucose load (2g/Kg) of male offspring of mice exposed to BPA (10 or 100 µg/kg/day) or vehicle on days 9 to 16 of gestation (n=8-10). (B) Ex vivo glucose-induced insulin secretion from isolated islets at 3, 7 and 16 mM glucose, from the same group of animals (C) $[Ca^{2+}]_i$ response of a representative islet of Langerhans from Control , BPA-10 and BPA-100 animals in the

presence of 3, 7 and 16 mM glucose applied for the period indicated by the bar. (n=10). (D) Area under the traces in C. (E) Measurement of β-cell area. The area occupied by insulin-positive cells is expressed as a percentage of total area. (F) Quantification of BrdU incorporation to insulin-positive cells. Grey column represents F1-C, black column F1-BPA10 and white column F1-BPA100 group respectively. *$p < 0.05$,. Data are expressed as mean±S.E.M.







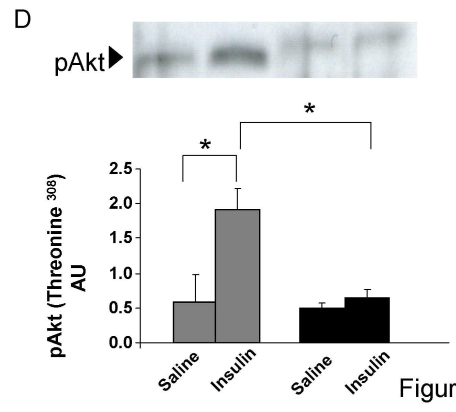


Figure 1

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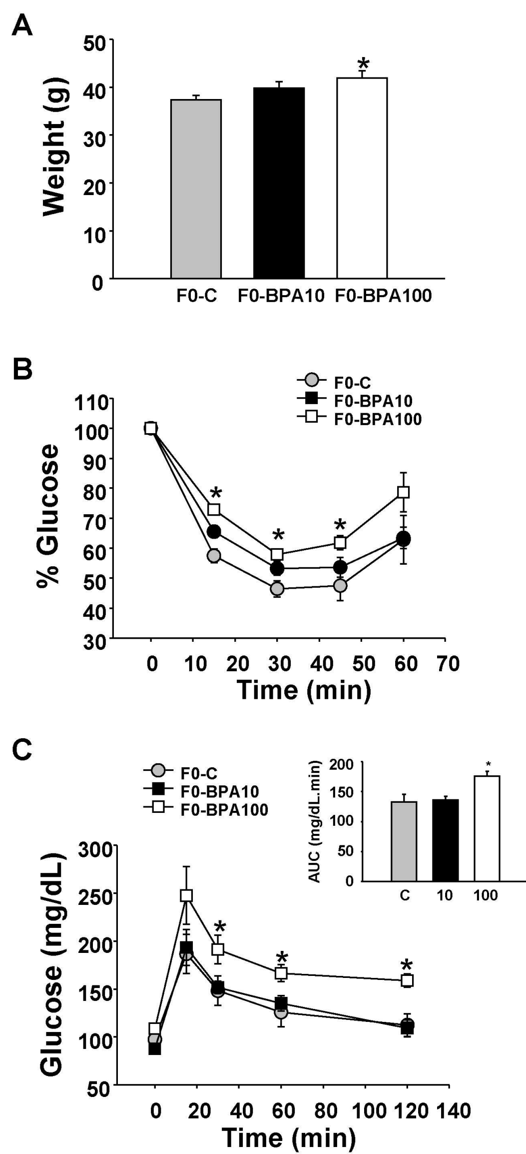

Figure 2

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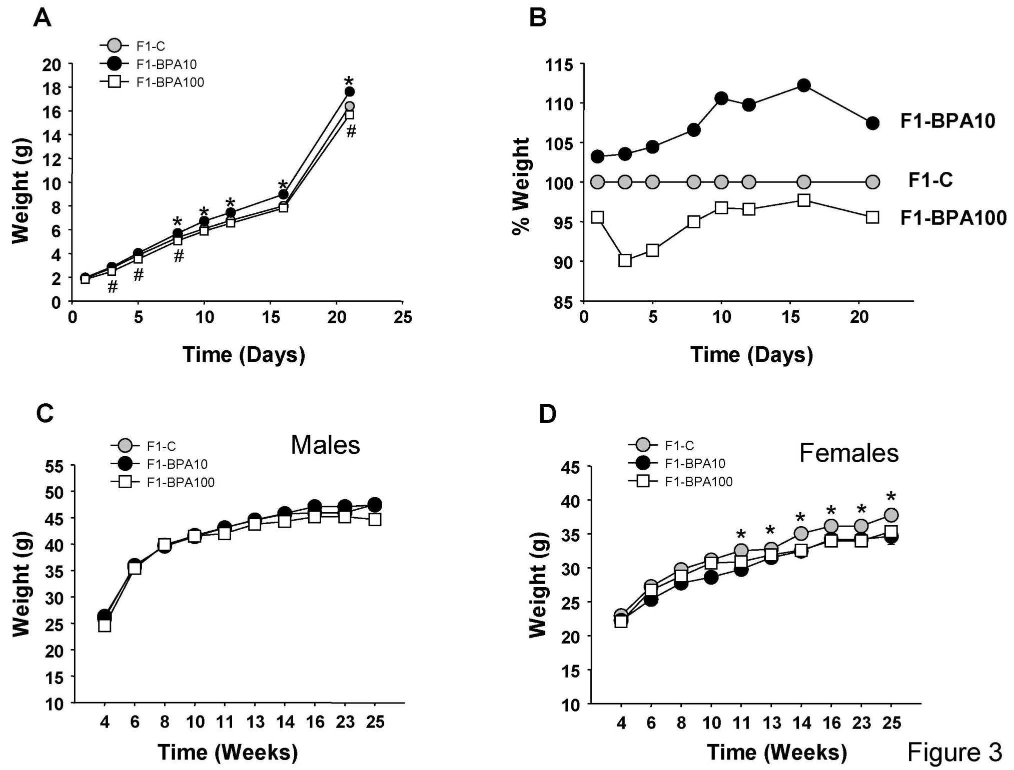

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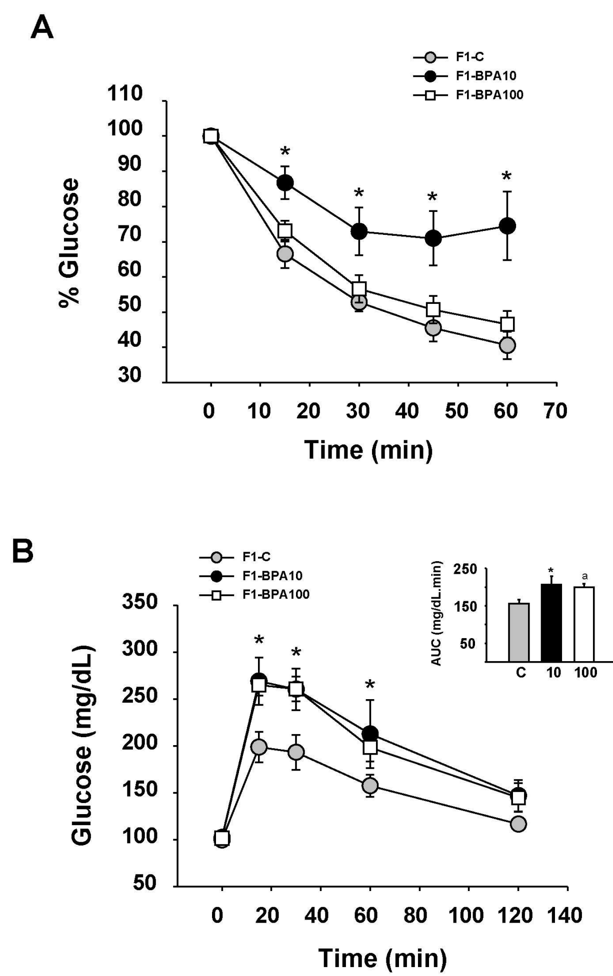

Figure 4

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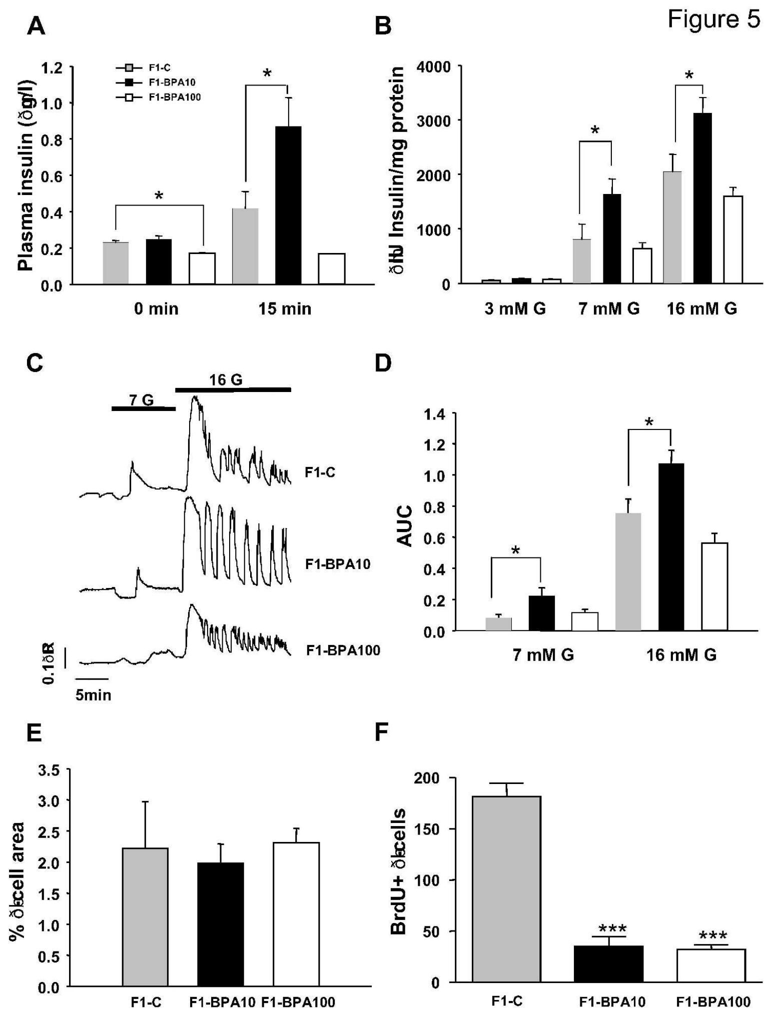

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