Neonatal Exendin-4 Treatment Reduces Oxidative Stress and Prevents Hepatic Insulin Resistance in Intrauterine Growth Retarded Rats

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ABSTRACT

Intrauterine growth retardation (IUGR) has been linked to the development of type 2 diabetes in adulthood. We have developed an IUGR model in the rat whereby the animals develop diabetes later in life. Previous studies demonstrate that administration of the long-acting glucagon-like-peptide-1 agonist, Exendin-4, during the neonatal period prevents the development of diabetes in IUGR rats. IUGR animals exhibit hepatic insulin resistance early in life (prior to the onset of hyperglycemia), characterized by blunted suppression of hepatic glucose production (HGP) in response to insulin. Basal HGP is also significantly higher in IUGR rats. We hypothesized that neonatal administration of Exendin-4 would prevent the development of hepatic insulin resistance. IUGR and control rats were given Exendin-4 on day 1-6 of life. Hyperinsulinemic-euglycemic clamp studies showed that Ex-4 significantly reduced basal HGP by 20% and normalized insulin suppression of HGP in IUGR rats. While Ex-4 decreased body weight and fat content in both Control and IUGR animals, these differences were only statistically significant in Controls. Exendin-4 prevented development of oxidative stress in liver and reversed insulin-signaling defects in vivo, thereby preventing the development of hepatic insulin resistance. Defects in glucose disposal and suppression of hepatic glucose production in response to insulin were reversed. Similar results were obtained in isolated Ex-4 treated neonatal hepatocytes. These results indicate that exposure to Exendin-4 in the newborn period reverses the adverse consequences of fetal programming and prevents the development of hepatic insulin resistance.

Key Words: Exendin-4, Intrauterine growth retardation, diabetes, liver, insulin resistance
**Abbreviations**: Ex-4: Exendin-4; IUGR: intrauterine growth retardation
INTRODUCTION

Uteroplacental insufficiency limits availability of substrates to the fetus and retards growth during gestation (40, 47). We have previously shown in a rat model of uteroplacental insufficiency and intrauterine growth retardation (IUGR) that this abnormal metabolic intrauterine milieu affects the development of the fetus by inducing mitochondrial dysfunction and oxidative stress which in turn modifies gene expression and function of susceptible cells in the pancreas, muscle, and liver (43, 48, 46). The end result is the later development of diabetes in adulthood with the salient features of most forms of type 2 diabetes (T2DM) in the human: defects in insulin action and insulin secretion (49). IUGR animals exhibit hepatic insulin resistance early in life (prior to the onset of hyperglycemia), characterized by blunted suppression of hepatic glucose production (HGP) in response to insulin (62) Basal HGP is also mildly elevated in IUGR rats (62). These defects in hepatic glucose metabolism are secondary to oxidative stress, which impairs insulin signaling (43, 62).

We have also demonstrated that short-term administration of the long-acting incretin hormone glucagon-like peptide-1 (GLP-1) receptor agonist, Exendin-4 (Ex-4), during the neonatal period improves glucose tolerance, prevents the progressive reduction in β-cell mass that is observed in IUGR rats, and dramatically prevents the development of diabetes in IUGR rats (50). Ex-4 shows a 53% amino acid sequence identity to GLP-1 (18) and shares many actions with GLP-1 via the GLP-1 receptor (37). Ex-4 stimulates glucose-dependent insulin response (23, 42, 31, 21, 65) suppresses glucagon secretion (31), and inhibits gastrointestinal motility (17). Ex-4 also regulates food intake (52). In addition to Exendin-4’s effects on the β-cell, it is also possible that Exendin-4 improves
glucose tolerance in IUGR rats via its extra-pancreatic effects, namely in the liver. Thus, in this study we tested the hypothesis that Ex-4 ameliorates oxidative stress, which in turn prevents the development of hepatic insulin resistance in IUGR rats.

**MATERIALS AND METHODS**

*Animal Model*

Our surgical methods have previously been described (40, 47). In brief, time-dated Sprague-Dawley pregnant rats (Charles River Laboratories, Wilmington, MA) were individually housed under standard conditions and allowed free access to standard rat chow and water. On day 19 of gestation, (term is 22 days), the maternal rats were anesthetized with intra-peritoneal xylazine (8mg/kg) and ketamine (40mg/kg), and both uterine arteries were ligated (IUGR). Rats recovered within a few hours and had *ad libitum* access to food and water. Animals were allowed to deliver spontaneously and litters were culled to 7-8 at birth to assure uniformity of litter size between IUGR and control litters. Only males were studied and when possible females were culled from the litters at birth. The remaining ratio of females and males in each litter was kept uniform. Each litter was considered an n of 1 and data from each litter was pooled.

All principles of laboratory animal care (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw references/phspol.htm) were followed, These studies were approved by the Animal Care and Use Committee of the Children’s Hospital of Philadelphia, the University of Pennsylvania, and the Albert Einstein College of Medicine.

*Neonatal Exendin-4 Treatment*
Exendin-4 was purchased from Bachem (King of Prussia, PA), prepared as a 1µM stock in 0.9% sodium chloride, and stored at -80°C in single use aliquots. Just prior to injection, aliquots were thawed and diluted in 1% BSA in 0.9% sodium chloride. Ex-4 (1 nmole/kg body weight) or vehicle (1% BSA in 0.9% saline) was injected subcutaneously daily for 6 days starting on day 1 of life. Four experimental groups were studied: 1) Control pups treated with vehicle; 2) Control pups treated with Ex-4; 3) IUGR pups treated with vehicle; and 4) IUGR pups treated with Ex-4.

**Body Composition**

Body composition was measured at 7-9 weeks of age by DEXA (Lunar Corporation, Madison WI). The DEXA scanner was specialized for small animals. The instrument settings used were as follows: a scan speed of 40 mm/s, a resolution of 1.0 X 1.0 mm, and automatic/ manual histogram width estimation. The coefficient of variation, as assessed by 3 repeated measurements (with repositioning of the rat between each measurement), was less than 5% and a total of 7 animals from different litters from each group were studied.

**Liver triglyceride Content**

Liver triglycerides were measured by a triglyceride kit (GPO- Trinder Sigma Diagnostics, St. Louis, MO, USA). Liver tissue was obtained from 7-9 week old male rats and snap frozen and stored at -80°C until use. Seven animals all from different litters in each group were studied. Frozen liver tissue (200 mg) was homogenized in 4 ml of 2:1 chloroform methanol. The solution was vortexed and filtered. After adding 0.2 ml of
0.58% NaCl solution, the filtrate was centrifuged (Sorvall 6000B) at 1000 rpm for 5 min.
The upper phase was aspirated. The chloroform phase was further analyzed. The sample
was evaporated in chloroform to a volume of less than 1 mL. Chloroform was added to
make up a final volume of 1 mL. Chloroform-containing samples, water, or standard
solution of glycerol (10–20 µl) were added to the cuvettes. Reagent (1 mL) was added.
After incubation at 30°C for 10 min, the samples were read in a spectrophotometer at 540
nm.

Hyperinsulinemic Euglycemic Clamp Studies

Hyperinsulinemic euglycemic clamp studies were performed as previously
described (62). In brief, six to seven male rats (from different litters) in each treatment
group were studied during young adulthood (300-400 g) at 7-9 weeks of age and were
normoglycemic. Animals were housed in individual cages and were subjected to a
standard light (6:00 AM to 6:00 PM)-dark (6:00 PM to 6:00 AM) cycle. One week before
the in vivo study, rats were anesthetized by inhalation of methoxyflurane and indwelling
catheters were inserted into the right internal jugular and left carotid artery (62). All
studies were performed after 6 hours of fasting while the animal was awake and
unstressed.

Rats received a primed continuous insulin infusion of 3mU.kg⁻¹.min⁻¹ to obtain
physiologic, post-meal, insulin levels. The animals also received a variable infusion of
25% dextrose that was periodically adjusted to clamp the plasma glucose concentration at
the basal level (euglycemic levels of 140 mg/dL) for the 2 hour period of the clamp study
and somatostatin (1.5 µg/kg/min) to inhibit endogenous insulin and glucagon secretion
Glucose levels were measured every 10 minutes and the rate was periodically adjusted to clamp the plasma glucose concentration at ~7.5 mM or 120-150 mg/dl. A primed, continuous infusion of high-performance liquid chromatography (HPLC)-purified [3-3H]-glucose (DuPont NEN, Boston, MA) [10 µCi bolus, followed by 0.05 µCi/min (basal) and 0.1 µCi/min (clamp)] was initiated at time 0 and maintained for 4 hours to estimate insulin-stimulated whole-body glucose flux and to calculate hepatic glucose production (HGP).

Rates of basal and clamp glucose turnover were determined as the ratio of the 3[H]-glucose infusion rate to the specific activity of plasma glucose at the end of the basal period and during the final 40 minutes of the clamp. HGP was calculated as the difference between the tracer-derived rate of appearance and the infusion rate of glucose. Regression analyses of the slopes of 3H2O Ra (used in the calculation of the rates of glycolysis) were performed at 30-minute intervals. The rate of glycolysis was estimated from the rate of conversion of 3[H]-glucose to 3H2O as previously described (62). Plasma-tritiated water specific activity was determined by liquid scintillation counting of the protein free supernatant (Somogyi filtrate) before and after evaporation to dryness.

**PEPCK and G6-Pase mRNA**

At 7 days and 7-9 weeks of age, total RNA was isolated from liver (n=5, all from different litters, per group) using RNAzol B (Tel-Test, Inc. TX). Quantitative PCR’s were carried out using equivalent dilutions of each cDNA sample, the fluorescent indicator SYBR green, the empirically determined concentration of each primer, and the Applied Biosystems model 7700 sequence detector PCR machine (PerkinElmer Life Sciences) as
previously described (62). To verify that only a single PCR product was generated for each amplified transcript, the multicomponent data for each sample was subsequently analyzed using the Dissociation Curves 1.0 program (PerkinElmer Life Sciences). To account for differences in starting material, quantitative PCR was also carried out for each cDNA sample using the Applied Biosystems human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 20x primer and probe reagent (PerkinElmer Life Sciences). GAPDH mRNA levels in liver did not differ between the four groups (p=0.23). The relative abundance of the target was divided by the relative abundance of GAPDH in each sample to generate a normalized abundance. Each reaction was carried out in triplicate. Standard PCR conditions were used.

Glucose-6-phosphatase and Glucokinase activity

G-6-Pase activity was assayed in intact microsomes prepared from liver 4 hours after the insulin clamps (n=5 animals from different litters for each group). Frozen liver was pulverized under liquid nitrogen, homogenized in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES) and 0.25 M sucrose, pH 7.4. Activity was assessed at glucose-6-phosphate concentrations of 1, 2.5, and 10 mM.

Glucokinase activity was determined at 7-9 weeks of age by the method described by Bontemps (7) with 100 mg of liver tissue. Liver was homogenized in 50 triethanolamine, 5 MgCl₂, 1 DTT, and 5 EDTA, pH adjusted to 7.5. The homogenate was centrifuged at 10,000g at 4°C. The supernatant was saved for the activity assay. The spectrophotometric assay of glucose phosphorylating activity was performed at two glucose concentrations: at 100 mM (measures all hexokinases including glucokinase) and at 0.5 mM (measures only the low Km hexokinases).
The difference between the two assays gives glucokinase activity. One unit is the amount of enzyme that catalyzes the formation of 1 µM of substrate/min, in the conditions of the assay.

**Hepatocyte Cell Culture**

Hepatocytes were isolated and pooled from a litter of 1-day-old control and IUGR newborn rats by collagenase digestion and cultured, as described by Gruppuso (12). The experiment was performed in 5 different litters. This method ensures greater than 90% purity and > 80% viability (by trypan blue exclusion) (12). Hepatocytes were cultured for 24 hours under standard conditions and then cultured for an additional 18 h in the presence of Exendin-4 (10nM).

**In Vitro Glucose Production**

After 24 hours in culture, the medium was removed and cells were cultured for an additional 18 hours in Ex-4 as described above. The cells were then incubated in glucose-free Hanks-Hepes medium containing 10 mmol/l pyruvate for 3 hours. The glucose released into the medium was determined enzymatically with glucose oxidase. The incubation medium was removed and hepatocytes were washed three times with cold saline and frozen immediately in liquid nitrogen. The amount of protein was determined by Lowry.

**Insulin signaling**

Changes in insulin signaling were determined as previously described (62). In brief, liver was harvested from 7-9 week-old animals (n = 5 animals from different litters
for each treatment group) after an overnight fast, snap frozen, and stored at -80°C prior to
western blot analysis as described below. To determine if Ex-4 treatment reversed defects
in insulin signaling in IUGR liver in a separate group of 7-week-old animals (n = 5 for
each treatment group), after ketamine (40 mg/kg) and xylazine anesthesia (40 mg/kg), 2
U/kg of insulin was injected into the portal vein. Five minutes after injection, the liver
was excised and frozen immediately. For experiments in hepatocytes, cells were treated
with insulin (100 ng/mL) for 5 minutes. Lysis buffer was added to the culture plate to
stop the reaction and cells were collected for further analysis.

Tissue or cells were disrupted by sonication, and homogenates were incubated with either
anti IRS2 or anti Akt overnight with protein A agarose beads. Immunoprecipitates were then
collected and washed and subjected to reducing SDS-PAGE using 12% Tris-glycine gels.
Proteins were electroblotted from the gels onto polyvinylidene difluoride membranes. The blots
were then incubated with tyrosine phosphorylated IRS2 (PY-IRS2) antibody (Upstate
Biotechnology, Lake Placid, NY). The membranes were stripped and then reprobed for anti-
IRS2 to measure the corresponding protein. To determine phosphorylation and abundance of Akt
in liver, homogenates were immunoprecipitated with Akt antibody (Cell Signaling Technology,
Beverly, MA) overnight with protein A agarose beads. Akt phosphorylation was determined on
Serine474, as described above. The membranes were stripped and reprobed with anti Akt
antibody. Protein bands were detected using enzyme-catalyzed chemiluminescence (ECL)
mediated by horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Images
were analyzed and bands quantified using MacBasv2.4 software (FujiPhoto Film, Tokyo, Japan).
Membranes were stripped and reprobed with β-actin as a protein loading control. Protein
phosphorylation was calculated as the ratio of phospho- to total protein expression.
**Western Blot Analysis**

Liver protein was isolated from at 7-9 weeks of age (n=5 from different litters in each group) and was subjected to reducing SDS-PAGE using 12% Tris-glycine gels. Proteins were electroblotted from the gels onto polyvinylidene difluoride membranes and probed with a polyclonal manganese superoxide dismutase (MnSOD) antibody (1:50,000) (StressGen, Ann Arbor, MI) followed by goat anti-rabbit horseradish peroxidase conjugated secondary antibody. Enzyme catalyzed chemiluminescence (ECL) mediated by horseradish peroxidase was developed with the ECL kit from Amersham Pharmacia Biotech (Piscataway, NJ) and detected with Super RX X-ray film from Fuji Medical Systems (Stamford, CT).

**Reduced (GSH) and Oxidized (GSSG) Glutathione Levels**

GSH and GSSG were assayed in frozen liver tissue from animals at 7-9 weeks of age (n=5 from different litters in each group) using a fluorescence plate reader and a kit (BioVision, Mountain View, CA). In the assay, OPA (o-phthalaldehyde), reacts with GSH (not GSSG), generating fluorescence, so GSH can be specifically quantified. Adding a reducing agent converts GSSG to GSH, so (GSH + GSSG) can be determined. To measure GSSG specifically, a GSH quencher is added to remove GSH, preventing reaction with OPA (while GSSG is unaffected). Reducing agent is then added to destroy excess quencher and to convert GSSG to GSH.
Assessment of Thiobarbituric Acid-Reactive Substances (TBARS)

Liver samples (25 mg) from each treatment group at 7 days of age (after Ex-4 treatment) and at 7-9 weeks of age were analyzed via the Cayman assay kit (Cayman, Ann Arbor, Michigan). Briefly samples (n=5 from different litters in each group) were sonicated in the presence of protease inhibitors and centrifuged at 1600 g for 10 min. One hundred µL supernatant was mixed with an equal volume of SDS solution and incubated in a water bath at 95°C for 1 hour. Then, the samples were centrifuged at 1600 g for 10 min and the TBARS concentration was spectrophotometrically measured at 530 nm against a saline blank. The TBARS was determined from the standard curve that was constructed with malondialdehyde and was expressed as nmol/mg protein. The amount of protein was determined by the Bradford method.

Analytical Procedures

At 7-9 weeks of age, glucose, insulin, nonesterified fatty acid (NEFA) and leptin levels were obtained in the fasted state (6 hours). In those animals not undergoing clamp studies, blood was collected from the tail vein. In animals undergoing clamp studies, blood was collected from the arterial catheter just prior to the study. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Inc., Palo Alto, CA). Plasma insulin, glucagon, and leptin levels were measured in duplicate by radioimmunoassay (Penn Diabetes Core at the University of Pennsylvania). NEFA concentrations were determined by an enzymatic method with an automated kit (Waco Pure Chemical Industries, Osaka, Japan).
Statistical Analysis

The significance of differences among groups was examined using ANOVA analysis and Tukey-Kramer for post-hoc analysis. All values are presented as means ± SE. A $p$ value of $<$0.05 was considered significant. All statistical analyses were performed using StatView software (5.01).
RESULTS

In vivo experiments

Effects of Ex-4 on Weight and Body Composition

Ex-4 treatment did not reduce weight of either Control or IUGR animals during the newborn treatment period (Figure 1). At 2 weeks and 7-9 weeks of age, Ex-4 reduced body weight in Control animals and IUGR animals. However, due to the variability in weights at these ages, the differences between Ex-4 and vehicle treated IUGR animals did not reach statistical significance (p<0.07) (Table 1). Similarly, Ex-4 significantly reduced fat mass in Control animals, but the reduction in fat mass in Ex-4 treated IUGR animals did not reach statistical significance (p<0.08).

Despite the well-known suppressive effect of GLP-1 and Exendin-4 on appetite (34), food intake was not affected by Exendin-4 treatment and daily caloric intake was similar among all groups between weaning (3 weeks) and 8 weeks of age (approximately 21-24 g/rat/day). As weights were not affected by Ex-4 treatment in the newborn period, it is unlikely that food intake was altered during the study period.

Metabolic Profile:

At 2 weeks of age (data not shown) and at the time of study (7-8 weeks of age), there were no significant differences in concentrations of glucose, glucagon, NEFA, or leptin levels among any of the groups (Table 2). However, as previously reported (50), fasting plasma concentrations of insulin were higher in IUGR compared to controls (p<0.01) and Ex-4 treatment significantly reduced insulin levels in both IUGR and
Control rats (p <0.05) (Table 2). There were no significant differences in hepatic triglyceride levels among any of the treatment groups (Table 2).

_Hepatic glucose production (HGP)_

Basal HGP was significantly higher in IUGR compared to Control vehicle treated rats (18.8 ± 1.8 vs. 15.25 ± 1.2 mg • kg⁻¹ • min⁻¹, p<0.05, in IUGR and Control, respectively). Ex-4 significantly reduced basal HGP in IUGR (from 18.8 ± 1.8 to 15.5 ± 0.8 mg • kg⁻¹ • min⁻¹, p<0.05 IUGR vehicle vs. IUGR Ex-4) but not in Control animals (15.25 ± 1.2 vs. 14.94 ± 1.4 mg • kg⁻¹ • min⁻¹, p = 0.2, Control vehicle vs. Control Ex-4).

During the clamp studies, the steady state plasma insulin levels were similarly increased to physiologic postprandial levels in all groups (68 ± 7, 65 ± 5, 63 ± 6, 66 ± 7 µU/ml in Control vehicle, Control Ex-4, IUGR vehicle, and IUGR Ex-4, respectively). Steady-state plasma glucose levels were also similar in all groups (148 ± 3.7, 146 ± 3.0, 148 ± 4.5, 142 ± 4.9 mg/dl in Control vehicle, Control Ex-4, IUGR vehicle, and IUGR Ex-4, respectively). HGP was suppressed from baseline in response to physiologic hyperinsulinemia in Control vehicle, Control Ex-4 and IUGR Ex-4. In contrast, IUGR vehicle treated animals had a significantly blunted response to insulin relative to the other 3 groups (Figure 2). IUGR vehicle treated animals suppressed HGP by 10%, compared to 36% in Control vehicle treated animals. IUGR animals that received neonatal Ex-4 treatment demonstrated normal suppression of HGP in response to insulin, with a 35% decrease in HGP. Furthermore, the glucose infusion rate (GIR) required to maintain normoglycemia in IUGR vehicle treated rats was ~40% lower than Controls (5.6 ± 0.7 vs. 9.5 ± 1.3 mg • kg⁻¹ • min⁻¹ in IUGR and Control, p<0.05). This decrease in the rate of
glucose infusion was largely accounted for by a lack of suppression of HGP. Ex-4 treatment normalized the GIR in IUGR’s (9.84 ± 0.4 mg • kg⁻¹ • min⁻¹). However, Ex-4 treatment did not modify the GIR in Control animals (9.5 ± 2.5 mg • kg⁻¹ • min⁻¹).

PEPCK and G-6-Pase mRNA

Previous studies showed a marked increase in PEPCK and G-6-Pase expression in IUGR liver both in newborn and adult animals (62). To determine whether Ex-4 altered PEPCK and G-6-Pase expression in IUGR animals, we measured mRNA levels at the end of Ex-4 treatment (day 7) and at 7-9 weeks of age by real-time PCR. At the end of Ex-4 treatment, PEPCK expression was 3.1± 0.2 and G-6-Pase was 2.2 ± 0.2 fold higher in vehicle IUGR liver compared to vehicle control liver (p<0.05, n = 7 for each group). Ex-4 treatment of IUGR animals completely normalized PEPCK and G-6-Pase mRNA levels such that there were no statistically significant differences between Ex-4 IUGR and Control vehicle or Control Ex-4 animals (p>0.1, n =7 for all 4 groups). This effect persisted into adulthood and PEPCK and G-6-Pase mRNA levels in Ex-4 IUGR animals were similar to Control Ex-4 and Control vehicle animals, whereas PEPCK expression was nearly 4 fold higher (3.8 ± 0.5) and G-6-Pase was 2.8 ± 0.2 fold higher in IUGR vehicle animals (p<0.05 vs. Control vehicle, Control Ex-4, and IUGR Ex-4, n = 5 animals each group).

G-6Pase and Glucokinase activity

As previously shown (62), despite a significant increase in G-6Pase mRNA levels, basal activity was not elevated in IUGR compared to control rats and Ex-4 had no effect on activity
(Table 3). G-6Pase and glucokinase activity three hours after administration of insulin also did not differ among treatment groups (Table 3).

**Insulin mediated glucose uptake**

In young prediabetic animals during physiological hyperinsulinemia, the rate of glucose uptake (Rd) was similar in all groups (Control Vehicle 21 ± 0.4; Control Ex-4 21 ± 0.4; IUGR Vehicle 21 ± 0.6; IUGR Ex-4 22 ± 0.4 mg • kg⁻¹ • min⁻¹). Furthermore, glycogen synthesis (Rd-glycolysis) and whole body glycolysis did not differ among groups (data not shown).

**Insulin signaling**

Basal levels of IRS-2 protein did not differ among the treatment groups, however IRS2 tyrosine phosphorylation (PY-IRS2) was significantly decreased in liver of 7-9-week-old IUGR vehicle treated animals compared to vehicle treated Controls (Figure 3). Further, after administration of insulin, there was no increase in tyrosine phosphorylation of IRS2 in IUGR vehicle treated animals. However, insulin induced a 2-fold increase in PY-IRS2 in liver of IUGR Ex-4 and both Control groups of animals (Figure 3). Both basal levels of Akt and insulin stimulated phosphorylated 473Ser-Akt protein levels were significantly decreased in IUGR vehicles compared to IUGR Ex-4, Control vehicle and Control Ex-4 (Figure 3).
Ex-4 effects on oxidative stress in liver

Our earlier studies indicated that IUGR induced oxidative stress in liver of offspring and that oxidative stress is ongoing and slowly increases with age (43). Thus we examined whether Ex-4 treatment protects neonatal liver against IUGR induced oxidative damage. Both acute and chronic production of reactive oxygen species (ROS) are often associated with large increases in the level of MnSOD protein, suggesting that mitochondria have mounted antioxidant defenses (10). Western blot analysis of liver protein from 7 day-old (3.2 ± 0.3 fold higher, p<0.05 vs. IUGR vehicle vs. Control vehicle) and 7-9 week old animals showed that the amount of MnSOD protein was significantly higher in IUGR compared to control animals and Ex-4 treatment completely prevented this increase in IUGR animals (Figure 4).

To further characterize the effects of Ex-4 on the condition of oxidative stress in the livers of IUGR and control rats we measured the levels of reduced and oxidized mitochondrial glutathione at 8 weeks of age. Figure 5 shows that the ratio GSH/GSSG was 30% lower in the IUGR compared to control vehicle treated animals (p<0.05), whereas the ratio of GSH/GSSG in IUGR Ex-4 treated animals was similar to that of control vehicle and control Ex-4 rats.

Thiobarbituric acid reactive substances (TBARS) are a sensitive measure of lipid peroxidation, a major indicator of oxidative stress. At the end of treatment, Ex-4 treated IUGR neonatal rats exhibited a significant decrease in hepatic TBARS concentration compared with vehicle treated IUGR pups (Figure 6a). This effect persisted into adulthood such that there were no significant differences in levels of TBARS among
Control vehicle, Control Ex-4, and IUGR Ex-4 treated animals compared to a nearly 2-fold elevation in TBARS in IUGR vehicle animals (Figure 6b).

**In Vitro Experiments**

**Insulin Signaling**

Despite the fact that many investigators have failed to detect GLP-1 receptor expression in liver, Ex-4 has been shown to increase intracellular levels of cAMP and phosphorylated CREB, PKB, PKCζ, and Erk1/2 in isolated hepatocytes (Aviv). These data suggest that Ex-4 treatment activates in liver cells a signaling pathway that is characteristic to GLP-1R signaling pathway. Thus, we sought to determine whether the effects of Exendin-4 treatment upon insulin signaling were mediated through a direct effect on the liver. Hepatocytes were isolated from control and IUGR one-day old newborn rats and treated for 18h with Exendin-4. Basal levels of IRS2 and Akt2 were not altered by Ex-4 treatment in either group. However, Ex-4 treatment modestly increased IRS2 and Akt2 phosphorylation in neonatal control hepatocytes and markedly increased IRS2 and Akt2 phosphorylation in IUGR hepatocytes (Figure 7).

**Hepatic Glucose Production**

Despite the absence of the Glp-1 receptor in liver, Ex-4 treatment significantly reduced PEPCK and G-6-Pase expression in isolated hepatocytes, from IUGR newborn animals. However, there was no change in expression in control cells (Figure 8a). Likewise, Ex-4 significantly decreased HGP only in IUGR hepatocytes (Figure 8b).
**DISCUSSION**

The major findings of our study were that a short treatment course of the GLP-1 receptor agonist, Exendin-4, in the newborn period prevented the development of hepatic insulin resistance in the adult IUGR animal and improved mitochondrial function and reduced oxidative stress. The effect of Ex-4 on glucose homeostasis is long-lasting with a resultant increase in the life span of IUGR animals (50).

Uteroplacental insufficiency induces oxidative stress in fetal liver which creates a self-perpetuating process whereby overproduction of ROS elicits mitochondrial dysfunction inducing further production of ROS creating a vicious cycle (43). Ex-4 treatment in the early newborn period prevents this cycle of gradually escalating and sustained stress which is associated with normalized hepatic insulin signaling in the IUGR rat. Ex-4 may ameliorate oxidative stress in newborn IUGR rats by improving glucose homeostasis (50). Even subtle elevations in glucose *in vivo* can increase the production of ROS by mitochondria. By facilitating normalization of glucose tolerance in early life, a time of rapid cellular expansion and differentiation, the self-reinforcing cycle of progressive deterioration in mitochondrial function is interrupted, thereby resulting in a long-lasting improvement in hepatic glucose homeostasis. It is also possible that Ex-4 has a direct effect on mitochondrial function. It has recently been reported that Ex-4 treatment of INS-1 cells (a β-cell line) prevents ROS production and mitochondrial dysfunction in response to cytokine induced oxidative stress (53). Further, prolonged exposure to reactive oxygen species has been shown to down-regulate IRS-2 and Akt phosphorylation *in vitro* (53, 29, 35, 33).
As previously reported, basal HGP was mildly increased in IUGR animals. Inappropriate HGP could be due to either increased flux through glucose-6-phosphatase and/or decreased flux through glucokinase. Because glucokinase activity, glucose-6-phosphatase activity, glycolysis, and glycogen content were both normal in IUGR animals, it is likely that increased gluconeogenesis is responsible for the observed increase in HGP in IUGR rats. A key step in gluconeogenesis is the formation of phosphoenolpyruvate from oxaloacetate, which is catalyzed by phosphoenolpyruvate carboxykinase (PEPCK). Recent studies have demonstrated that overexpression of PEPCK alone can increase hepatic glucose production (51, 58). A two-fold elevation, similar to the magnitude of change observed in IUGR animals, results in a 30% increase in basal HGP but normal plasma glucose levels (58). Other data suggest that gluconeogenesis is regulated by activities of multiple enzymes in combination with PEPCK (24, 5, 10). Indeed, the regulation of flux between the multiple pathways involved in glucose metabolism in the liver plays a crucial role in maintaining metabolic homeostasis. It is plausible, therefore, to speculate that in individuals destined to become diabetic, increased flux through the PEPCK pathway precedes the development of overt hyperglycemia. Fasting hyperglycemia develops in the IUGR animal once either hepatic glucose production increases beyond a certain threshold, β-cell compensation fails, peripheral glucose disposal decreases, or any combination of the above.

Our finding that Ex-4 treatment decreased expression of PEPCK in IUGR liver at the end of treatment was surprising, as cAMP is known to increase PEPCK transcription. It is possible that a chronic elevation in cAMP levels (as mediated by chronic Ex-4 administration) may not induce PEPCK expression. The mechanisms responsible for the reduction in PEPCK levels in Ex-4 IUGR animals are more likely to be secondary to
prevention of oxidative stress, which has been shown to lead to elevated PEPCK expression (29).

It is generally believed that Ex-4 mediates a majority of its actions via interaction with the GLP-1 receptor (54, 22). Most investigators have been unable to detect significant levels of GLP-1 receptor in adult rat liver (8, 16, 6, 11). In agreement with these previous studies, we were unable to convincingly detect GLP-1 receptor protein or mRNA in neonatal liver (data not shown). To date, only one GLP-1 receptor has been identified (6, 11, 14, 15, 25, 36, 64, 63). However there are studies in which some actions of GLP-1 are not blocked by the antagonist Exendin-(9-39) (38, 13) suggesting that Ex-4 may mediate some of its actions through a different receptor. Our data demonstrating that Ex-4 treatment of cultured primary neonatal hepatocytes decreases PEPCK expression and normalizes insulin signaling supports this hypothesis. Further, two groups have reported that GLP-1 has insulin-like activity and stimulates glycogen formation (59) and inhibits glycogenolysis (27) in isolated hepatocytes. These investigators suggest that GLP-1 action in the liver is mediated by receptors that are different in structure and signaling pathways (38, 59, 27, 20, 26, 32, 56). It is also possible that Ex-4 is mediating its actions in the IUGR neonate through GLP-1 receptors in the portal vein as a number of investigators have shown that Ex-4 can reduce glucose independent of islet hormones or gastric emptying (38, 41, 3, 4, 9, 28, 39, 57).

Regardless of the type of receptor that Ex-4 mediates its effects in the liver, it is clear that neonatal treatment with Ex-4 has profound and long-lasting effects on insulin action in the liver in IUGR animals that are mediated through a reduction in oxidative stress.

Until recently, it had been assumed that the glucoregulatory effects of GLP-1
were solely due to its ability to regulate the secretion of pancreatic hormones (41, 1, 2, 45, 55, 60, 61). However, studies using Glp1r knockout (Glp1r−/−) mice clearly demonstrate that glucoregulatory properties of GLP-1 are both insulin dependent and insulin independent (44). These data show that GLP-1 regulates hepatic and muscle glucose flux independent of its ability to enhance insulin secretion. Further, it is also possible that Ex-4 mediates its effects on hepatic glucose metabolism through a central effect. It has recently been shown that a gut-glucose sensor modulates peripheral glucose metabolism through a nutrient-sensitive mechanism, which requires brain GLP-1 receptor signaling (30). It remains to be determined whether the effects of Ex-4 on hepatic glucose metabolism and insulin signaling in IUGR rats are mediated primarily through the β-cell or are independent of its effect on pancreatic hormone secretion, however our studies in isolated neonatal hepatocytes suggest that Ex-4’s hepatic effects may be directly mediated.

In summary, the long-term improvement in hepatic glucose metabolism after neonatal Ex-4 treatment suggests that there may be a unique opportunity to influence the development of adult onset diabetes in humans by intervening during the pre-diabetic period in at-risk individuals.

**Acknowledgements**

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56. Trapote MA, Clemente F, Galera C, Morales M, Alcantara AI, Lopez-Delgado MI, Villanueva-Penacarrillo ML, Valverde I. Inositolphosphoglycans are possible


Table 1: **Body Weights and Body composition.** For weights, values from 7-8 animals per litter were pooled and 7 litters in each group were studied. Body composition was measured by Dexa scans and one to two animals per litter were studied, n = 7 litters for each group. Data are expressed as means ± SEM. *p<0.05 Control vehicle vs. IUGR vehicle and IUGR Ex-4 rats; (g= grams); † p <0.05 Control Veh vs. Con Ex-4 rats.

<table>
<thead>
<tr>
<th></th>
<th>Con Vehicle</th>
<th>Con Ex-4</th>
<th>IUGR Vehicle</th>
<th>IUGR Ex-4</th>
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</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2 weeks</td>
<td>31.2 ± 0.29*</td>
<td>28.12 ± 0.61†</td>
<td>25.13 ± 3.99</td>
<td>24.16 ± 2.66</td>
</tr>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>8 weeks</td>
<td>410 ± 28*</td>
<td>328 ± 31†</td>
<td>386 ± 34</td>
<td>330 ± 34</td>
</tr>
<tr>
<td>Visceral fat (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body mass 2 weeks</td>
<td>15.1 ± 0.05*</td>
<td>13.8 ± 0.08†</td>
<td>16.1 ± 0.09</td>
<td>15.2 ± 0.09</td>
</tr>
<tr>
<td>Visceral fat (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body mass 8 weeks</td>
<td>8.9 ± 1.1*</td>
<td>7.9 ± 1.1†</td>
<td>9.9 ± 1.0</td>
<td>8.4 ± 0.8</td>
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</table>

Table 2: **Basal metabolic characteristics at 7-9 weeks of age.** Blood was obtained from tail vein after an overnight fast. Data are expressed as means ± SEM. *p<0.05 IUGR Vehicle vs. Control Vehicle; †p<0.05 IUGR Vehicle vs. IUGR Ex-4, **p<0.05 Control Ex-4 vs. Con Veh. Values from 7 animals each from a different litter in each group were studied.

<table>
<thead>
<tr>
<th></th>
<th>Con Vehicle</th>
<th>Con Ex-4</th>
<th>IUGR Vehicle</th>
<th>IUGR Ex-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>144.7 ± 7.4</td>
<td>142.4 ± 5.2</td>
<td>147.2 ± 6.3</td>
<td>152.1 ± 6.3</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>13.82 ± 0.41</td>
<td>11.67 ± 0.36**</td>
<td>18.06 ± 1.07**†</td>
<td>10.74 ± 1.04</td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>0.64 ± 0.02</td>
<td>0.58 ± 0.01</td>
<td>0.40 ± 0.03</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>312 ± 49</td>
<td>299 ± 46</td>
<td>325 ± 56</td>
<td>312 ± 69</td>
</tr>
<tr>
<td>Leptin (µg/L)</td>
<td>3.3 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td>3.7 ± 0.4</td>
<td>3.1 ± 0.5</td>
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<tr>
<td>Liver Triglyceride</td>
<td>7.8 ± 1.3</td>
<td>7.0 ± 1.2</td>
<td>8.3 ± 1.8</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>(mg/gram protein)</td>
<td></td>
<td></td>
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</tbody>
</table>
Table 3. Hepatic G-6Pase (mU/mg protein) and glucokinase activities (mU/mg protein) in Ex-4 and Vehicle treated rats. Intact microsomes were prepared and activity of enzymes assayed as described in "Methods". One animal per litter (n=5 litters) was studied. Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control Vehicle</th>
<th>Control Ex-4</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Insulin</td>
<td>Basal</td>
</tr>
<tr>
<td>G-6-Pase</td>
<td>2.84 ± 0.31</td>
<td>1.01 ± 0.01</td>
<td>3.36 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>4.19 ± 0.79</td>
<td>5.33 ± 1.2</td>
<td>5.15 ± 0.66</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>4.23 ± 0.56</td>
<td>4.89 ± 0.81</td>
<td>4.89 ± 0.69</td>
</tr>
</tbody>
</table>

|                      | IUGR Vehicle    | IUGR Ex-4    |                      |
|                      | Basal           | Insulin      | Basal               |
| G-6-Pase             | 2.99 ± 0.66     | 0.81 ± 0.02  | 2.41 ± 0.33         |
|                      | 4.23 ± 0.56     | 4.89 ± 0.81  | 3.99 ± 1.78         |
Figure 1. Weights of newborn rats during treatment period. Weights were averaged for a litter. Each litter contained 7-8 animals. IUGR vehicle (n=7 litters), IUGR Ex-4 (n=7 litters), Control vehicle (n=6 litters), and Control Ex-4 treated rats (n=6 litters). Values are means ± standard error of the mean. * p < 0.05 IUGR vehicle vs. Control vehicle and Control Ex-4.

Figure 2. Hepatic Glucose Production at 7-9 weeks of age. Basal hepatic glucose production and suppression of hepatic glucose production during insulin infusion (3 mU/ Kg/min) in IUGR vehicle (n=7 animals each from a different litter), IUGR Ex-4 (n=7, each from a different litter), Control vehicle (n=6, each from a different litter), and Control Ex-4 treated rats (n=6, each from a different litter) at 7-8 weeks of age. Values are means ± standard error of the mean. * p < 0.05 basal vs. insulin stimulation; ** p < 0.05 IUGR vehicle vs. Control vehicle; † p < 0.05 IUGR vehicle vs. IUGR Ex-4.

Figure 3. Insulin Signaling in Liver at 7-9 weeks of age. A. Representative immunoblots. B. Quantification of insulin signaling proteins. Rat liver protein was isolated from in IUGR vehicle (IV), IUGR Ex-4 (IE), Control vehicle (CV), and Control Ex-4 (CE) treated rats before and after insulin administration into the portal vein and western and immunoblot analyses were carried out using IRS-2, PY-IRS-2, Akt, p-Akt as described in the Methods. Data are expressed as fold increase in phosphorylation and as means ± SEM of values from 5 rats (each from a different litter) in each group. *p<0.05 IV vs. CV, CE, and IE.

Figure 4. MnSOD in Liver at 7-9 weeks of age. A. Representative western blot. B. Quantification of MnSOD proteins. Rat liver protein was isolated from IUGR vehicle
(IV), IUGR Ex-4 (IE), Control vehicle (CV), and Control Ex-4 (CE) treated rats and western blot analyses were carried out using MnSOD antibody as described in the Methods. Data are expressed as arbitrary units and as means ± SEM of values from 5 rats (each from a different litter) in each group. *p<0.05 IV vs. CV, CE, and IE.

**Figure 5. GSH/GSSG Ratios in Liver at 7-9 weeks of age.** Liver was isolated from IUGR vehicle (IV), IUGR Ex-4 (IE), Control vehicle (CV), and Control Ex-4 (CE) treated rats and GSH and GSSG levels measured as described in the Methods. Data are expressed as means ± SEM of values from 5 rats (each from a different litter) in each group. *p<0.05 IV vs. CV, CE, and IE.

**Figure 6. TBARS in Liver.** TBAR levels in liver at the end of treatment at 7 days of age (4a) and at 7-8 weeks of age (4b). Liver was isolated from IUGR vehicle (IV), IUGR Ex-4 (IE), Control vehicle (CV), and Control Ex-4 (CE) treated rats and TBAR levels measured as described in the Methods. Data are means ± SEM of values from 5 rats (each from a different litter) in each group. *p<0.05 IV vs. CV, CE, and IE.

**Figure 7. Insulin signaling in newborn hepatocytes.** Hepatocytes were isolated from IUGR and Control newborn rats (n=5 different litters) and cultured for 24 hours as described in the Methods. Hepatocytes were then treated for 18 hours with Ex-4 (10nM). Cells were harvested to determine basal levels of IRS-2 and Akt or were treated with insulin (100 ng/mL) for 5 minutes and western and immunoblot analyses were carried out using IRS-2, PY-IRS-2, Akt, p-Akt as described in the Methods. Data are expressed as fold increase in phosphorylation and as
means ± SEM of values from 5 experiments in each group. ** p<0.01 IUGR Vehicle (IV) vs. Control Vehicle (CV).

**Figure 8. Glucose production in newborn hepatocytes.** A) Hepatocytes were isolated from IUGR and Control newborn rats (n=5 different litters) and cultured for 24 hours as described in the Methods. Hepatocytes were then treated for 18 hours with Ex-4 (10nM) and harvested for quantification of mRNA levels for PEPCK and G-6-Pase via q-PCR. B) Hepatocytes isolated from newborn rats were cultured as described above and then incubated in glucose-free Hanks-Hepes medium in the presence of 10 mmol/L pyruvate and treated with 80 nmol/L of insulin. Data are expressed as means ± SEM for 5 separate experiments. *p<0.05 IV vs. CV, CE, and IE.
Figure 4

A.

MnSOD

B.

Bar graph showing MnSOD activity across different groups (CV, CE, IE, IV).
Figure 5

[Bar chart showing GSH/GSSG levels for CV, CE, IE, IV groups]