Effects of Prenatal Alcohol Exposure on Glucose Tolerance in the Rat Offspring

Li Chen and B.L. Grégoire Nyomba

Low birth weight in humans predisposes to obesity, cardiovascular diseases, and type 2 diabetes in adult life. Alcohol exposure during pregnancy has been associated with fetal growth restriction. We investigated the effects of prenatal exposure to alcohol on glucose metabolism later in the offspring. Female Sprague Dawley rats were given ethanol (ETOH), 4 g/kg/day by gavage throughout pregnancy. Compared with controls, newborn ETOH rats had decreased body size (5.1 ± 0.1 *v* 6.3 ± 0.1 g, *P* < .001), plasma insulin (0.44 ± 0.4 *v* 0.67 ± 0.1 ng/mL, *P* < .05), and leptin mRNA (*P* < .05), but they had normal β-cell mass and elevated adipose resistin mRNA and plasma glucose (5.0 ± 0.5 *v* 3.6 ± 0.3 mmol/L, *P* < .01). Food intake was decreased in ETOH rats during the fourth week of life, and body weight remained decreased compared with controls until a catch-up growth occurred by 7 weeks of life. At 13 weeks of age, body weight and β-cell mass of ETOH offspring were normal, but plasma glucose and insulin after a glucose challenge were increased compared with controls (*P* < .05). Adipose leptin and hypothalamic Ob-R mRNA were not different from controls, but resistin was increased (*P* < .05), and muscle GLUT4 content was decreased (*P* < .05) in ETOH offspring compared with controls. The data suggest that prenatal alcohol exposure impairs glucose tolerance in the offspring by both inducing insulin resistance and β-cell dysfunction. The prevailing mechanism in 3-month-old rat offspring appears to be insulin resistance, manifested by glucose intolerance and decreased GLUT4 despite hyperinsulinemia.

ADVERSE EVENTS during pregnancy may interfere with fetal physiology, metabolism, and development and may program the fetus to adulthood diseases. In epidemiologic studies, intrauterine growth restriction (IUGR) has been associated with insulin resistance, impaired glucose tolerance, type 2 diabetes, coronary heart disease, and hypertension later in life.[1-4] Other studies have found associations between intrauterine exposure to the diabetic milieu and obesity, insulin resistance, and glucose intolerance in the offspring.[5,6] Similar results have been reported with regard to prenatal exposure to famine, which causes IUGR.[7,8] Animal models of IUGR have generally supported these epidemiologic studies. The best studied model uses a low-protein diet during pregnancy.[9,10] Offspring with IUGR in this model have increased insulin sensitivity in young adult life, but they may develop glucose intolerance during senescence.[11] Global food restriction during pregnancy and lactation in rats results in IUGR and the offspring, although remaining small, progressively develop insulin resistance and glucose intolerance.[12,13] Global undernutrition may also be induced surgically by uterine artery ligation, resulting in placental ischemia. The adult offspring in this model develop obesity, insulin resistance, and diabetes.[14]

Besides these nutritional models, hormones and toxins have also been used to study the impact of intrauterine and perinatal events on adulthood diseases. Prenatal exposure to excess glucocorticoids resulted in IUGR and cardiovascular diseases in the adult.[15] Maternal endotoxemia induced by bacterial lipopolysaccharides caused IUGR and was associated with obesity and insulin resistance in adult offspring.[16] Female rats developed insulin resistance and male-type changes in body fat distribution during adulthood after imprinting with 1 dose of testosterone during the perinatal period.[17] These studies suggest that abnormal events in utero and in early life may be deleterious to fetal growth and may program the fetus to diseases in later life.

Alcohol ingestion during pregnancy can lead to abnormal fetal development, sometimes manifested as the fetal alcohol syndrome (FAS)[18] IUGR is a hallmark of FAS, and the prevalence of FAS is elevated in populations with lower socioeconomic status,[19,20] where type 2 diabetes is also common.[21,22] It is unclear, however, if alcohol abuse during pregnancy is commonly associated with glucose intolerance in the adult offspring. The present study was undertaken to determine to what extent alcohol exposure during pregnancy leads to the development of glucose intolerance in the rat offspring.

MATERIALS AND METHODS

Animals and Experimental Design

Timed-pregnant Sprague-Dawley rats were housed in individual cages under controlled temperature, humidity, and light cycle and were allowed free access to tap water and commercial rat chow (Agway Prolab, Syracuse, NY) providing a balanced amount of minerals and vitamins, 3.5 kcal/g metabolizable energy, and containing by weight 22.5% proteins, 5.5% fat, and 62% carbohydrates. The rats were randomly divided into 2 weight-matched groups. Throughout gestation, 1 group (ethanol [ETOH]) was given ETOH 2 g/kg (36%) by gavage twice daily at 9 AM and 4 PM, and the second group (control) was given the same volume of water instead of ETOH. Body weight and food intake were recorded from day 14 of gestation to parturition. The offspring studied were from 6 dams per group. Litter size per dam was 16.3 ± 1.5 in ETOH and 17.3 ± 1.5 in controls (*P* = not significant [NS]). On the first day of life, 20 pups per group (day-1) were killed by decapitation, trunk blood was collected in heparinized capillary tubes, and used for glucose determination. Pools of plasma from 4 pups each were used in the insulin assay (see below). Brain, subcutaneous fat, and

From the Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada.

Submitted May 20, 2002; accepted October 23, 2002.

Supported by grants from the Canadian Diabetes Association, the Manitoba Medical Services Foundation, and the H.E. Sellers Foundation.

Address reprint requests to B. L. Grégoire Nyomba, MD, PhD, Health Sciences Centre, University of Manitoba, 820 Sherbrook St, Rm GG449, Winnipeg, Manitoba, Canada R3A 1R9.

0026-0495/03/5204-0012$30.00/0

doi:10.1053/meta.2003.50073

Table 1. Primer Sequences and Position

Primer	Sequence	Position
Resistin sense	5′-TTTTCCTTTTCTTCCTTGTCC-3′	110-131
Resistin antisense	5′-TGCTGTCCAGTCTATCCTTGC-3′	376-355
GAPDH sense	5′-CCATGGAGAAGGCTGGG-3′	388-405
GAPDH antisense	5′-CAAAGTTGTCATGGATGACC-3′	582-563
Leptin sense	5′-TCACACACGCAGTCGGTAT-3′	165-184
Leptin antisense	5′-TTCAGGGCTAAGGTCCAAC-3′	528-509
Ob-R sense	5′-CTCCGCACTCACAGGCAACA-3′	297-317
Ob-R antisense	5′-GGTGGATCGGGCTTCACAA-3′	717-698

pancreas were rapidly dissected out, snap frozen in liquid nitrogen, and stored at −70°C. The remaining rats (6 to 7 per lactating dam) were kept with their mothers until weaning on day 21. Weaned offspring were housed 3 per cage and fed a normal chow. For body weight and food studies, the rats were housed 1 day per week in individual plastic cages with metal wire basket tops. Food was weighed and placed on the basket top. After 24 hours, the remaining food was weighed, and food intake was calculated as the difference between the 2 weights, corrected for any food spill assessed by scanning the cage bedding. Body weights were recorded at the same time. At 13 weeks of age, 6 offspring in both the ETOH and control groups were fasted overnight and underwent an intraperitoneal glucose tolerance test (IPGTT) by 9 AM the next morning. Glucose (30%wt/vol), 2 g/kg body weight, was injected intraperitoneally, and tail blood (40 μL) was collected at 0, 30, 60, and 120 minutes for glucose determination. The rats were killed by exsanguinations, and gastrocnemius muscle was stored at −70°C until used. Another set of 6 rats per group was killed without prior overnight fast and hypothalamus, gastrocnemius muscle, pancreas, and epididymal adipose tissues were obtained. Aliquots of plasma were stored at −20°C until assayed. The protocol was approved by the Committee for Animal Use in Research and Teaching of the University of Manitoba.

Blood Alcohol Determination

Blood alcohol was determined in a subset of 6 dams in the morning of day -20 of gestation before, 2 hours, and 4 hours after alcohol gavage. Offspring from these dams were not used. Alcohol level was determined spectrophotometrically using an alcohol dehydrogenase kit (Sigma, St Louis, MO).

Leptin, Leptin Receptor, and Resistin Expression

Total RNA was extracted from approximately 100 mg adipose tissue by the Trizol method (Life Technologies, Rockville, MD). The tissue was homogenized in 1 mL Trizol, and the homogenate was centrifuged at 12,000*g* for 10 minutes at 4°C. The supernatant was transferred to an Eppendorf tube to which 0.2 mL chloroform was added, and the centrifugation was repeated for 15 minutes. The aqueous phase was transferred to a fresh tube, and 0.5 mL isopropyl alcohol was added. After a third centrifugation for 10 minutes, the pellet, containing RNA, was washed once with 1 mL 75% ETOH. After centrifugation at 7,500*g* for 5 minutes at 4°C, the RNA pellet was dissolved in RNase-free water. RNA loading amounts were compared by ethidium bromide staining of 18S and 28S ribosomal RNA after formaldehyde-agarose gel electrophoresis.

The first-strand cDNA for resistin was synthesized from 5 μg total RNA using SuperScript reverse transcriptase and Oligo(dT) primers. A total of 5 μL of the reverse transcription product was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase and specific primers for resistin (Table 1). Another 5 μL of the reverse transcription product was amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Table 1) as an internal control. The conditions for PCR were 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 90 seconds (30 cycles). The expected RT-PCR product of resistin is 267 bp in length.

Reverse transcriptase (RT)-PCR for leptin and leptin receptor (OB-R) was performed with 1 μg total RNA from adipose and brain tissues, respectively, using the OneStep RT-PCR Kit (Qiagen, Valencia, CA). The cDNA was synthesized with Sensiscript Reverse Transcriptase and HotStarTaq DNA Polymerase (Life Technologies) and specific primers (Table 1) at 50°C. The conditions for PCR were 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute (30 cycles). The expected RT-PCR product for leptin is 363 bp in length. The expected product for OB-R is 420 bp in length. β-actin was used as an internal control. RT-PCR products (10 μL) were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and densitometrically analyzed using National Institutes of Health (NIH) Image software (Bethesda, MD).

Glucose Transporter-4 Content

Gastrocnemius muscle tissue (500 mg) was homogenized for 5 seconds using a Brinkman homogenizer (Westbury, NY) in ice-cold TES buffer (20 mmol/L Tris-HCl, pH 7.4, containing 250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 0.01 mmol/L leupeptin, and 5 μg/mL aprotinin). The homogenate was spun twice at 3,000*g* for 10 minutes at 4°C, the supernatant spun again at 100,000*g* for 90 minutes at 4°C, and the resultant precipitate suspended in ice-cold TES by shearing using 22, 25, and 30-gauge needles. Protein (50 μg per lane) was separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and electroblotted onto nitrocellulose membranes. Blots were blocked with 5% dry milk for 1 hour and incubated with rabbit anti-GLUT4 antiserum at 1:1,000 dilution for 1 hour at room temperature. Blots were then washed in TBS-Tween for 15 minutes, incubated with goat antirabbit horseradish peroxidase-conjugated secondary antibody at 1:3,000 for 1 hour at room temperature, and washed in TBS-Tween for 15 minutes. Immune complexes were detected using the ECL chemiluminescent detection kit before exposing the blots to a Kodak Biomax Light Film (Rochester, NY). GLUT4 protein was quantified by densitometry using NIH Image software.

Pancreatic Insulin Content

Pancreases were dissected and immediately frozen at −70°C. Proteins were extracted using the acid-ethanol method.[23] Briefly, pancreases weighing 350 to 450 mg were homogenized in 5 mL acid-ethanol buffer (1.5 mL HCl 12 mol/L in 100 mL 70% ETOH) and incubated overnight at 4°C for further extraction. On the next day, samples were centrifuged at 3,000*g* for 15minutes at 4°C, and supernatants were stored at −70°C for assay. Insulin content was measured with a rat insulin radioimmunoassay (RIA) kit.

Pancreatic Histomorphometry

The whole pancreas was dissected out, weighed, and fixed in 10% buffered formaldehyde solution. The tissue was dehydrated, embedded in paraffin, and 6-μm sections were stained with hematoxylin and eosin by standard procedures. Paraffin-embedded sections were also immunostained using the Dako Envision+ system (Mississauga, Canada) according to the manufacturer's recommendations. Sections were first deparaffinized with xylene and rehydrated with ETOH and distilled water. Sections were next incubated with a rabbit anti-insulin immunoglobulin (1:100), followed by peroxidase-labeled polymer conjugated to goat antirabbit immunoglobulin. Sections were then stained with 3,3′-diaminobenzidine substrate-chromogen, counterstained with hematoxylin and eosin, and examined by light microscopy for islet counting. Morphometric analysis was performed using an Nikon light microscope connected to a Nikon Microprojection System and North-

ern Exposure software (Hollywood, CA). The relative cross-sectional area of β cells was determined by marking the threshold of the captured image for brown tissue (β cells) and for blue tissue (exocrine pancreas). Three individuals, 2 of whom were blinded to the study, performed these analyses, and the means of the determinations were used. Total tissue area was corrected for the unstained area. β-cell density was calculated as the cross-sectional area of β cells per tissue section area. β-cell mass per pancreatic piece was estimated as the product of β-cell density and the weight of the fresh pancreatic piece, and total β-cell mass per animal was estimated from the fresh weight of the pancreas.

Other Assays

Plasma insulin and leptin were measured with rat-specific RIA kits. Plasma resistin was determined by enzyme immunoassay with an antibody raised against the human resistin(51-108)-NH_2, which recognizes rat resistin. Glucose was measured using YSI2300 glucose analyzer (YSI, Yellow Springs, OH). Tissue protein was determined by the Bradford method using bovine serum albumin as standard.

Materials

Rat insulin and leptin RIA kits were from Linco (St Charles, MO). Resistin enzyme immunoassay kit was purchased from Phoenix Pharmaceuticals (Belmont, CA). Antibodies for Western blots were from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin immunohistochemistry kit was from Dako Diagnostics (Mississauga, Ontario, Canada). Electrophoresis reagents were purchased from BioRad (Hercules, CA). ECL chemiluminescence kit was obtained from Amersham Pharmacia (Piscataway, NJ). Trizol, SuperScript reverse transcriptase, Taq DNA polymerase and Oligo(dT) primers were obtained from Life Technologies (Rockville, MD). The OneStep RT-PCR Kit was purchased from QIAGEN (Valencia, CA). cDNA primers (Table 1) were synthesized by Life Technologies, except for β actin, which was purchased from Clontech (Palo Alto, CA). Ethanol to be administered to rats was obtained from pharmaceutical services of the Health Sciences Centre (Winnipeg, MB, Canada). Isopropyl alcohol and methanol were from Fisher Scientific (Nepean, Ontario, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Statistics

All analyses were conducted with SPSS software (version 10.1 for Windows, SPSS, Chicago, IL). Differences between groups were evaluated by unpaired *t* test. Insulin values were log-transformed before analysis. Individual glucose and insulin measurements during IPGTT were compared by repeated measures analysis of variance. Values are expressed as the mean ± SEM. $P < .05$ was considered significant.

RESULTS

Animal Weights, Food Intake, and Leptin

Daily food intake (23.0 ± 1.4 g [80.5 ± 4.9 kcal] *v* 25.7 ± 1.0 g [89.9 ± 3.4 kcal]) during the last week of gestation was slightly lower ($P = .12$) in ETOH dams (n = 9) compared with controls (n = 7), but weight gain during pregnancy was similar (136 ± 15 *v* 138 ± 10 g, P = not significant [NS]). The amount of ETOH ingested (~1.5g/day) provided ~7 kcal/g or ~10.5 kcal/day, increasing the total caloric intake in ETOH dams to ~91.0 kcal/day. Alcoholemia at 0 hour, 2 hours, and 4 hours after ETOH gavage was 7.1 ± 1.4, 114.4 ± 26.9, and 70.0 ± 20.0 mg/dL, respectively. The period of gestation was similar between ETOH (21.5 ± 0.3 days [range, 20 to 22]) and control dams (21.3 ± 0.3 days [range, 21 to 22]). On the first day of life, ETOH pups weighed significantly less than controls (5.1 ± 0.1 *v* 6.3 ± 0.1 g, $P < 0.001$), and this weight difference persisted until 6 weeks of age (Table 2). ETOH rats then caught up to control weight. Epididymal fat pad weights were not different between ETOH and control groups (9.5 ± 0.6 *v* 10.9 ± 1.1 g, P = NS). To investigate whether the catch-up weight gain was due to overeating, we measured food intake weekly after weaning. Food intake was significantly lower in ETOH rats compared with controls at 4 weeks of age, but it reached control level by 5 weeks and subsequently increased with age similarly in both groups until 8 weeks and then plateaued (Table 2).

Table 2. Body Weight and Food Intake of ETOH and Control Rats

	Body Weight (g)		Food Intake (g/day)	
Week	Control	ETOH	Control	ETOH
0	6.3 ± 0.1	5.1 ± 0.1*		
3	79 ± 0.8	65 ± 0.6*		
4	148 ± 1.5	130 ± 3.2*	13.2 ± 0.3	11.4 ± 0.2*
5	208 ± 4.4	198 ± 1.4†	20.7 ± 0.3	19.8 ± 0.4
6	275 ± 2.4	245 ± 5.3*	24.1 ± 0.3	23.2 ± 0.5
7	316 ± 9.2	322 ± 4.7	27.5 ± 0.2	26.5 ± 0.8
8	393 ± 4.2	387 ± 6.0	30.0 ± 0.4	29.6 ± 0.6
9	426 ± 7.2	425 ± 5.4	30.3 ± 0.5	30.8 ± 0.6
10	473 ± 7.7	477 ± 8.3	29.2 ± 0.6	29.3 ± 0.4
11	501 ± 5.4	502 ± 5.6	30.1 ± 0.7	32.5 ± 1.1
12	517 ± 9.8	532 ± 5.8	29.9 ± 0.5	30.0 ± 0.6
13	555 ± 9.0	570 ± 7.7	30.3 ± 0.4	31.1 ± 0.4

NOTE. Weight is shown from day-1 to 13 weeks of age. Food intake was recorded from 4 to 13 weeks. Data shown as the mean ± SEM (n = 12 rats/group).

*$P < .001$, †$P < .05$, ETOH *v* control rats.

To examine the effect of intrauterine ETOH exposure on the leptin system, we measured subcutaneous adipose tissue leptin mRNA and brain Ob-R mRNA in day-1 rats. In 13-week-old animals, we measured circulating leptin, epididymal fat leptin mRNA, and hypothalamus Ob-R mRNA. Leptin mRNA was significantly lower in ETOH than in control animals on day-1, but not at 13 weeks of age (Fig 1). Ob-R mRNA levels were not different between ETOH and controls at the 2 age groups studied (Fig 2), and plasma leptin levels were similar between adult ETOH rats and controls (8.1 ± 1.0 *v* 7.1 ± 0.7 ng/mL, P = NS).

Glucose Tolerance, Insulin, GLUT4, and Resistin

To study the effect of ethanol exposure on glucose homeostasis, we measured plasma glucose and insulin levels in the nonfasting state in day-1 and 13-week-old rats and during an IPGTT after an overnight fast in 13-week-old animals. In day-1 rats, glucose levels were significantly higher (5.0 ± 0.5 *v* 3.6 ± 0.3 mmol/L, $P < .01$), and insulin levels were lower (0.44 ± 0.4 *v* 0.67 ± 0.1 ng/mL, $P < .05$) in ETOH rats than in controls. In 13-week-old rats, the nonfasting glucose levels were similar between ETOH and control animals (6.4 ± 0.2 *v* 6.0 ± 0.2 mmol/L, P = NS), but insulin levels were elevated in the ETOH rats (4.8 ± 0.5 *v* 2.5 ± 0.9 ng/mL, $P < .05$). These animals also had significantly higher plasma glucose and insulin levels after intraperitoneal glucose challenge (Fig 3).

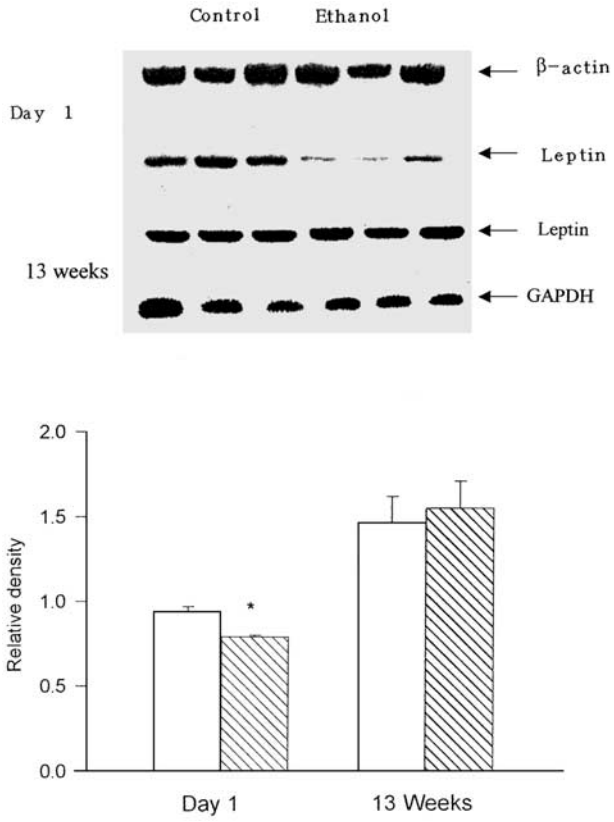

Fig 1. Leptin expression in ETOH and control rats. In day-1 rats (6/group) and in 13-week-old rats (6/group), leptin mRNA was measured in subcutaneous adipose tissue and in epididymal adipose tissue, respectively. Densitometric mRNA measurements, expressed in arbitrary units relative to β actin or GAPDH mRNA levels, are shown as the mean ± SEM. *$P < .05$, ETOH *v* control rats.

The area under the insulin curve was significantly greater in ETOH rats compared with controls (110 ± 14.2 *v* 78.5 ± 6.8 ng/mL · min, $P < .05$).

Because glucose intolerance in association with hyperinsulinemia suggests the presence of insulin resistance, we measured GLUT4 in skeletal muscle membranes in 13-week-old rats. In the random-fed state, GLUT4 level was not different between groups despite differences in circulating insulin levels. After glucose challenge, however, GLUT4 was significantly lower in ETOH animals (Fig 4). We also determined resistin mRNA in adipose tissue of day-1 and 13-week-old rats and found significantly elevated levels in ETOH rats compared with controls at both age groups (Fig 5). Plasma resistin levels, measured only in the adult group, were also higher in ETOH than in controls (3.54 ± 0.27 *v* 2.61 ± 0.26 ng/mL, $P < .05$).

Pancreas Morphometry and Insulin Content

Because changes in insulin levels in ETOH rats could be caused by β-cell changes, we measured pancreatic insulin content and performed histomorphometric analyses of pancreatic islets both on day-1 and at 13 weeks of age (Fig 6 and Table 3). On day-1, the pancreas was slightly heavier in the ETOH rats compared with controls, but it had a similar weight in 13-week-old rats. Pancreatic insulin content was significantly decreased in ETOH day-1 rats compared with controls, but it was similar between groups at 13 weeks of age. Figure 6 depicts representative pancreatic sections stained with hematoxylin and eosin to identify the islets (Fig 6A and B), or immunostained for insulin to identify βcells (Fig 6C and D). The number of islets per unit of exocrine pancreatic area was comparable between ETOH and control day-1 rats, but it was significantly greater in 13-week-old ETOH animals compared with controls (Table 3). At both ages, however, β-cell density and β-cell mass were not different between ETOH and control rats (Table 3). As expected, insulin content per unit pancreas and islet cell density were greater in newborn rats than in adult animals.

DISCUSSION

In this report, we have studied the role of alcohol ingestion by the mother during pregnancy on glucose homeostasis in newborn and adult offspring. On day-1, ETOH offspring were small and had decreased leptin mRNA and insulin levels, but a normal β-cell mass, elevated plasma glucose, and increased expression of adipose tissue resistin. The decreased insulin levels despite normal β-cell mass is an indication of β-cell immaturity.[24,25] The ETOH offspring displayed a catch-up growth within 7 weeks of life in association with increased

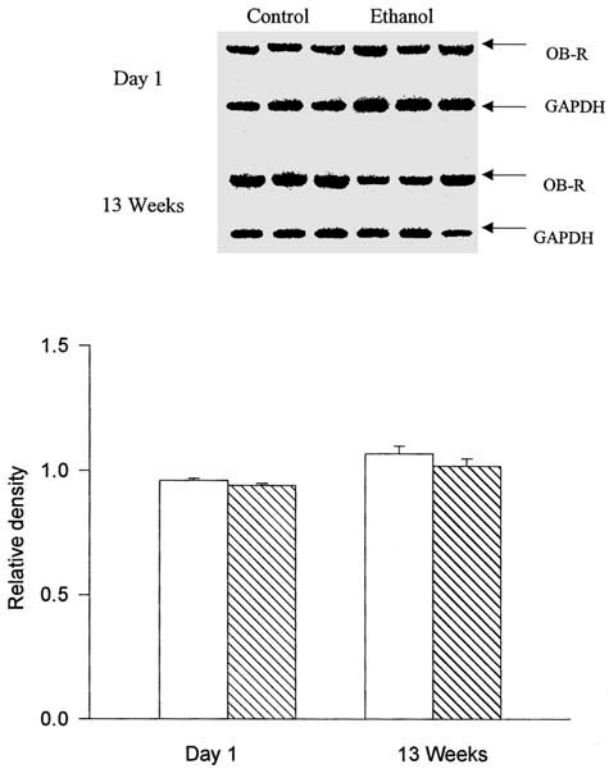

Fig 2. Ob-R expression in ETOH and control rats. In day-1 rats (6/group) and in 13-week-old rats (6/group), Ob-R mRNA was determined in the brain and in the hypothalamus, respectively. Densitometric mRNA determinations, expressed in arbitrary units relative to GAPDH mRNA levels, are shown as the mean ± SEM.

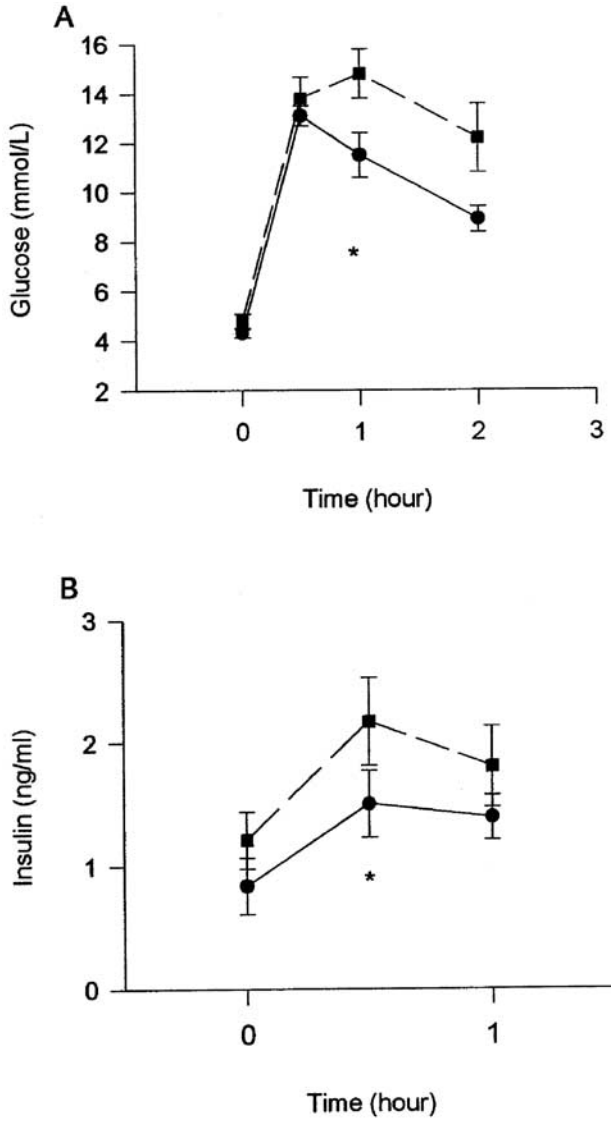

Fig 3. (A) Plasma glucose and (B) insulin levels during an IPGTT in 13-week-old ETOH (■) and control (●) rats. Individual glucose and insulin measurements were compared by repeated measures ANOVA. The area under the insulin curve, compared using a *t* test, was also significantly greater in ETOH rats compared with controls (110 ± 14.2 *v* 78.5 ± 6.8 ng/mL · min, $P < .05$). *$P < .05$, ETOH *v* control rats by repeated measures ANOVA.

food intake remarkable after 4 weeks of age. In 13 weeks old ETOH rats, adipose tissue weight, leptin mRNA, and circulating leptin concentrations were not different from controls, but adipose tissue expression of resistin was still elevated. At this adult age, the ETOH animals also had elevated insulin levels and decreased skeletal muscle GLUT4 content, and they developed impaired glucose tolerance. An early study in humans reported the presence of glucose intolerance with hyperinsulinemia in 3 of 7 children with FAS.[26] The development of glucose intolerance in the ETOH offspring despite hyperinsulinemia indicates the presence of insulin resistance.

Glucose transport across the plasma membrane is the rate-limiting step for glucose metabolism in skeletal muscle, and the insulin-dependent glucose transporter, GLUT4, is a primary determinant of insulin-stimulated glucose uptake and metabolism in this tissue.[27] Thus, increasing muscle GLUT4 content by transgenic overexpression or by increased contractile activity enhances insulin-stimulated muscle glucose uptake. Conversely, reducing the content of GLUT4 by gene knockout, denervation, or aging impairs insulin-mediated muscle glucose uptake.[28] The decreased skeletal muscle GLUT4 content in ETOH offspring, therefore, provides a mechanism for insulin resistance in these animals.

Resistin, a recently discovered protein also known as FIZZ3[29] or ADSF,[30] has been suggested to link obesity to type

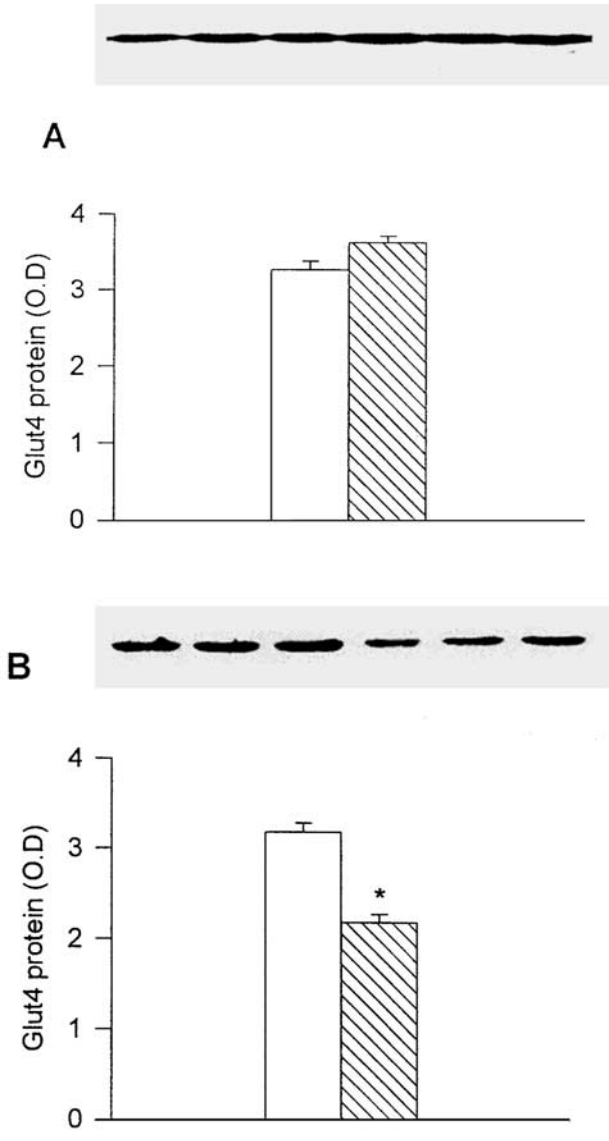

Fig 4. Skeletal muscle GLUT4 content in 13-week-old ETOH and control rats. Shown are representative GLUT4 immunoblots and densitometric analyses of the blots performed in the (A) random-fed rats, n = 5 per group and (B) after an intraperitoneal glucose tolerance test, n = 5 rats/group. *$P < .05$, ETOH *v* control rats.

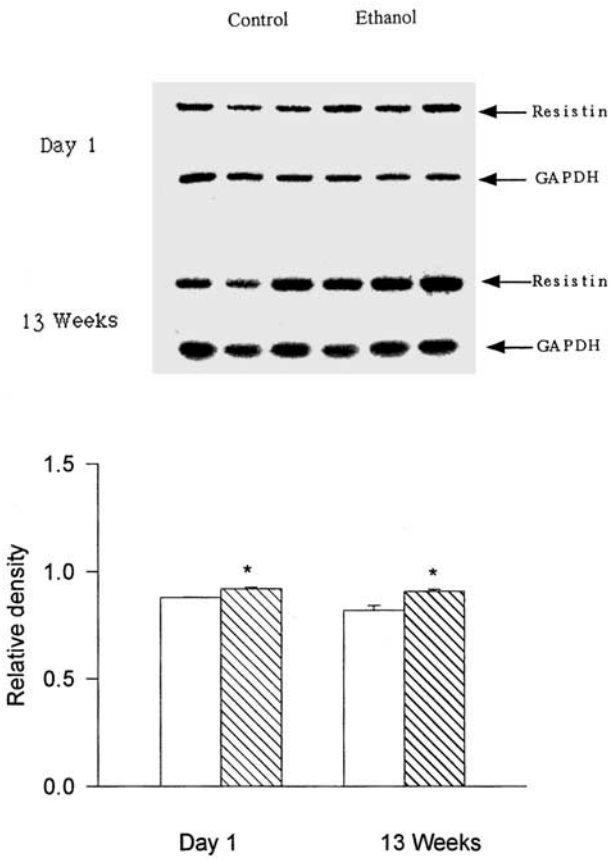

Fig 5. Resistin expression in adipose tissue of ETOH and control rats. Resistin mRNA were measured by RT-PCR in day-1 rats (6/group) and in 13-week-old rats (6/group) and expressed in arbitrary units relative to GAPDH mRNA levels. *$P < .05$, ETOH *v* control rats.

2 diabetes.[31] Resistin is expressed in adipocytes and secreted into the circulation. Steppan et al[31] found elevated resistin levels in both genetic (ob/ob and db/db) and diet-induced obese diabetic mice. In their study, antiresistin antibody improved blood glucose and insulin sensitivity in mice with diet-induced obesity, while administration of recombinant resistin impaired insulin action in vivo in mice and ex vivo in adipocytes. In addition, circulating resistin levels and adipocyte resistin gene expression were markedly decreased by treatment with the insulin sensitizer, antidiabetic drug, rosiglitazone.[31] Several studies have found elevated levels of resistin in association with obesity.[31-34] In insulin-resistant Fischer rats, resistin gene expression increased in proportion to weight gain, but not in relationship to insulin resistance, and it was suggested that resistin is a marker of adiposity.[34] Resistin expression may be regulated by nutrition status and insulin in rodents, because carbohydrate feeding increases resistin expression in fasted mice and rats, and insulin injection has a similar effect in streptozotocin diabetic mice.[30] In the current study, we found elevated resistin mRNA in adipose tissue from both newborn and adult ETOH rats. In addition, plasma resistin levels were elevated in ETOH adult offspring compared with controls. Adiposity is unlikely to explain the difference in resistin expression in our adult rats, because body weight and epididymal fat mass (an index of total body fat mass[35,36]) were similar between groups. Because ETOH newborn rats were smaller and hypoinsulinemic compared with controls, adiposity or insulin are also unlikely factors in the resistin difference at this age. This is the first report of resistin expression in an IUGR model. Although the exact role for resistin in insulin resistance is still unclear, a possible scenario in this model is that persistently elevated resistin from birth to adulthood interfere with insulin action resulting in decreased GLUT4, hyperinsulinemia, and glucose intolerance.

The ETOH rats in the current study had an increased growth rate within the first 7 weeks of life, but the mechanism of this phenomenon is unclear. On day 1, the ETOH offspring had a lower adipose tissue expression of leptin compared with controls. At 13 weeks of age, however, ETOH animals had similar levels of adipose leptin mRNA and circulating leptin compared with controls, at the time their weights were similar. These results are in agreement with previous reports that leptin synthesis and secretion is proportional to body weight.[37] It is noteworthy that although food intake was similar between rat groups, except at the fourth week, the ETOH group was smaller during the first 6 weeks of life and, then consumed proportionally larger amounts of food compared with controls. It is, therefore, possible that neonatal hypoleptinemia played a role in the growth spurt of ETOH rats through an increase in food intake, as suggested in humans born with IUGR.[38,39]

Maternal ETOH ingestion can cause IUGR via maternal malnutrition, placental malfunction, or direct fetotoxicity.[40] Maternal nutrition was provided in the current study through a balanced diet and although food intake of the ETOH dams was somewhat lower, daily caloric intake was similar to controls, explaining similar weight gain during pregnancy,[41] and protein intake was higher than what has been shown to cause dietary malnutrition. It has been suggested that alcohol may have direct growth delaying effects through inhibition of DNA and protein synthesis[42] and that alcohol can cause placental dysfunction resulting in reduced transport of amino acid and other nutrients to the fetus and in fetal malnutrition.[43,44]

Earlier reports indicated that fetuses of rats given ethanol-containing diets were hypoglycemic as a consequence of decreased glucose uptake by the placenta or fetal tissue.[45,46] In newborn offspring of rats given ethanol during pregnancy, insulin levels were increased while glucose levels were normal, suggesting a state of insulin resistance.[47] Studies in adult life have suggested that alcohol abuse can cause insulin resistance,[48,49] and ethanol has been shown to inhibit insulin-induced insulin receptor substrate-1 phosphorylation[50] and phosphatidyl inositol-3-kinase activity in vitro.[51] It is possible, therefore, that the metabolic and mitotic effects of prenatal alcohol exposure, which result in IUGR, affect pathways of glucose homeostasis preventing them from coping with metabolic stress later in life and resulting in impaired insulin sensitivity.

This study confirms the view that adverse events during pregnancy result in poor fetal growth and may have deleterious consequences during adulthood, as reported in epidemiologic studies,[1-4,7,8] and supported by animal models of IUGR.[9-16] The low-protein diet during pregnancy is a frequently used model of

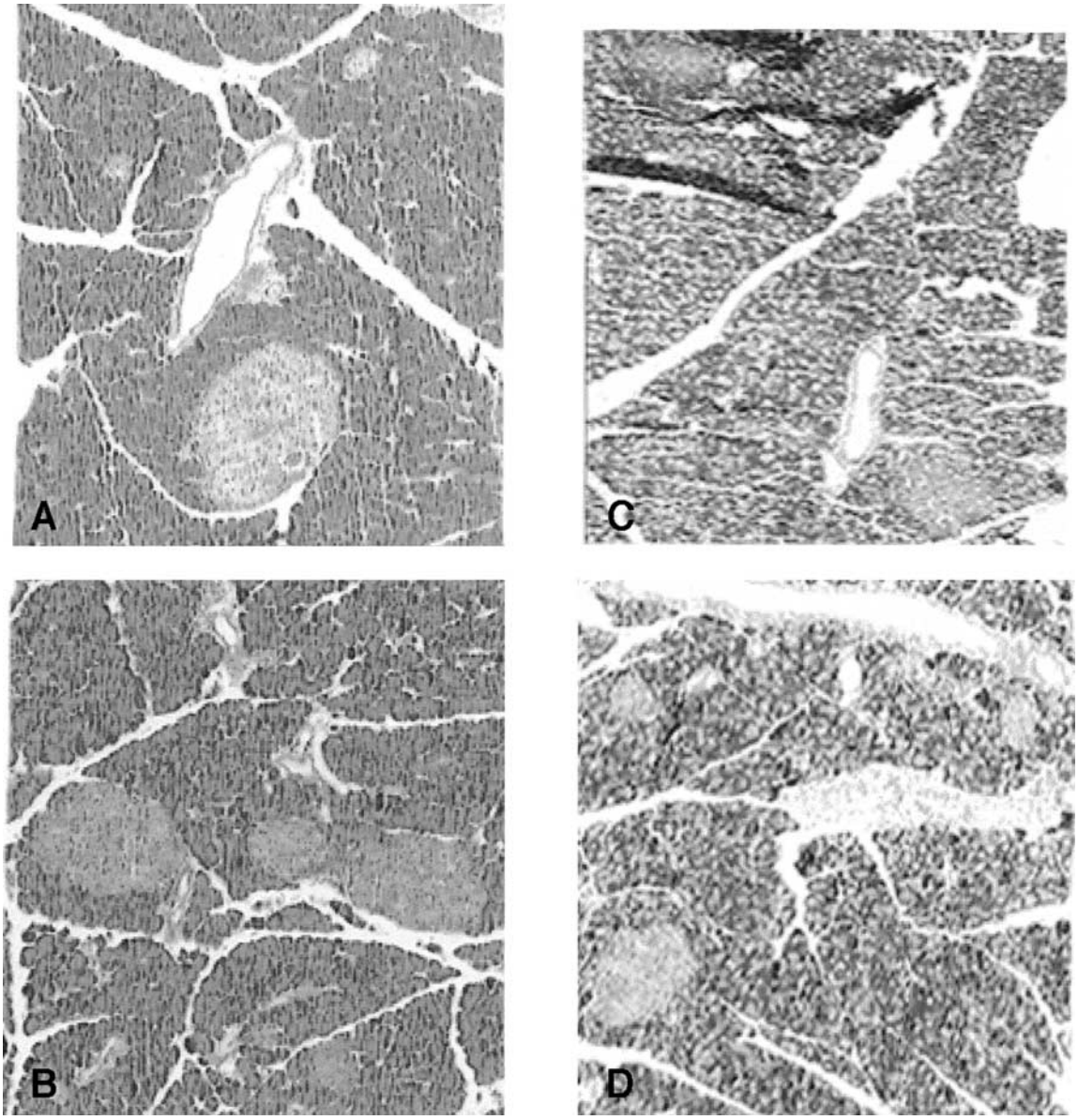

Fig 6. (A and B) Pancreatic sections stained with hematoxylin and eosin or (C and D) immunostained for insulin in control (A and C) and ETOH (B and D) 13-week-old rats.

IUGR.[9,10] The rat offspring in this model have a reduced β-cell mass throughout life. However, these animals have increased insulin sensitivity as young adults,[10] but they develop glucose intolerance with senescence.[11] A second model is that of global undernutrition, in which animals are submitted to food restriction.[12,13] The offspring of undernourished mothers are small at

Table 3. Insulin Content and Morphometry of the Pancreas in ETOH and Control Rats

	Day 1		13 Weeks	
	Control	ETOH	Control	ETOH
Pancreas weight (mg)	13 ± 0.5	16 ± 1.0*	828 ± 52	792 ± 83
Pancreas insulin (μg/mg protein)	2.26 ± 0.21	1.77 ± 0.05*	0.69 ± 0.04	0.82 ± 0.10
Islets/mm^2	12.7 ± 1.1	14.7 ± 1.5	0.9 ± 0.1	1.3 ± 0.1†
β-cell density	6.7 ± 1.3	6.4 ± 1.8	1.3 ± 0.3	1.8 ± 0.2
β-cell mass (mg)	0.8 ± 0.1	1.0 ± 0.3	11.2 ± 2.5	12.9 ± 2.6

NOTE. These studies were performed in day-1 rats (20/group) and 13-week-old rats (6/group).
*$P < .05$, †$P < .01$ ETOH *v* age-matched controls.

birth and when fed standard diet after weaning, they remain small despite being hyperphagic. They have a normal fasting plasma glucose, but increased fasting plasma insulin and leptin concentrations.[13] The elevated leptin levels, which are proportional to the retroperitoneal fat pad weights, are attributed to a state of leptin resistance thought to be responsible for the hyperphagia.[13] By 70 days of age, the offspring have decreased insulin release and β-cell mass and impaired glucose tolerance.[12] A third model uses bilateral uterine artery ligation to cause placental ischemia.[14] In this model, the offspring become obese and develop diabetes due to decreased β-cell mass and peripheral insulin resistance, as measured by insulin tolerance test. ETOH offspring developed signs of insulin resistance and impaired glucose tolerance in adulthood. Unlike the uteroplacental insufficiency and protein deprivation models, however, β-cell mass was not decreased in ETOH animals.

In humans predisposed to become diabetic, β cells first compensate for insulin resistance by increasing insulin output to maintain normoglycemia, and this phase is followed by a decompensation phase during which insulin secretion fails and overt hyperglycemia develops.[52,53] In the placental insufficiency model,[14] insulin secretion decreased in the offspring by 15 weeks of age, and this is the time overt hyperglycemia developed. The ETOH rats had increased islet number and insulin secretion at 13 weeks of age, suggesting that they were at a compensatory phase. It is possible that β-cell mass and insulin secretion in these rats would decrease if these animals were followed longer than 13 weeks.

In summary, the results of this study demonstrate that exposure to ETOH in utero results in impaired glucose tolerance in the offspring through insulin resistance and early insulin hyposecretion, which are key factors in the pathogenesis of type 2 diabetes. The prevailing mechanism in 3-month-old rat offspring appears to be insulin resistance.

REFERENCES

1. Barker DJ, Hales CN, Fall CH, et al: Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidemia (syndrome X): Relation to reduced fetal growth. Diabetologia 36:62-67, 1993
2. Valdez R, Athens MA, Thompson GH, et al: Birthweight and adult health outcomes in a biethnic population in the USA. Diabetologia 37:624-631, 1994
3. Lithell HO, McKeigue PM, Berglund L, et al: Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years. BMJ 312:406-410, 1996
4. Rich-Edwards JW, Colditz GA, Stampfer MJ, et al: Birthweight and the risk for type 2 diabetes mellitus in adult women. Ann Intern Med 130:278-284, 1999
5. Pettitt DJ, Bennett PH, Saad MF, et al: Abnormal glucose tolerance during pregnancy in Pima Indian women: Long-term effects on offspring. Diabetes 40:126-130, 1991 (suppl 2)
6. Silverman BL, Cho NH, Metzger BE: Impaired glucose tolerance in adolescent offspring of diabetic mothers: Relationship to fetal hyperinsulinism. Diabetes Care 18:611-618, 1995
7. Phillips DIW: Birth weight and the future development of diabetes: A review of the evidence. Diabetes Care 21:B150-B155, 1998 (suppl 2)
8. Ravelli ACJ, van der Meulen JHP, Michels RPJ, et al: Glucose tolerance in adults after prenatal exposure to famine. Lancet 351:173-177, 1998
9. Dahri S, Snoeck A, Reusens-Billen B, et al: Islet function in offspring of mothers on low-protein diet during gestation. Diabetes 40:115-120, 1991 (suppl 2)
10. Ozanne SE, Wang CL, Coleman N, et al: Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. Am J Physiol 271:E1128-E1134, 1996
11. Hales CN, Desai M, Ozanne SE, et al: Fishing in the stream of diabetes: From measuring insulin to the control of fetal organogenesis. Biochem Soc Trans 24:341-350, 1996
12. Martin MA, Alvarez C, Goya L, et al: Insulin secretion in adult rats that had experienced different underfeeding patterns during their development. Am J Physiol 272:E634-E640, 1997
13. Vickers MH, Breier BH, Cutfield WS, et al: Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. Am J Physiol 279:E83-E87, 2000
14. Simmons RA, Templeton LJ, Gertz SJ: Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. Diabetes 50:2279-2286, 2001
15. Benediktsson R, Lindsay RS, Noble J, et al: Glucocorticoid exposure in utero: A new model for adult hypertension. Lancet 341: 339-341, 1993
16. Nilsson C, Larsson BM, Jennische E, et al: Maternal endotoxemia results in obesity and insulin resistance in adult male offspring. Endocrinology 142:2622-2630, 2001
17. Nilsson C, Niklasson M, Eriksson E, et al: Imprinting of female offspring with testosterone results in insulin resistance and changes in body fat distribution at adult age in rats. J Clin Invest 101:74-78, 1998
18. Clarren SK, Smith DW: The fetal alcohol syndrome. N Engl J Med 298:1063-1067, 1978
19. Abel EL: An update on incidence of FAS: FAS is not an equal opportunity birth defect. Neurotoxicol Teratol 17:437-443, 1885
20. Bingol N, Schuster C, Fuchs M, et al: The influence of socioeconomic factors on the occurrence of fetal alcohol syndrome. Adv Alcohol Subst Abuse 6:105-118, 1987
21. Barker DJP, Gardner MJ, Power C: Incidence of diabetes amongst people aged 18-50 years in nine British towns: A collaborative study. Diabetologia 22:421-425, 1982
22. Marshall JA, Hamman RF, Baxter J, et al: Ethnic differences in risk factors associated with the prevalence of non-insulin dependent diabetes mellitus. The San Luis Valley Diabetes Study. Am J Epidemiol 137:706-718, 1993
23. Best HC, Haist RE, Ridout JE: Diet and the insulin content of the pancreas. J Physiol 97:107-119, 1939
24. Dudek RW, Kawabe T, Brinn JE, et al: Glucose affects in vitro maturation of fetal islets. Endocrinology 114:582-587, 1984
25. Hellerstrom C, Swenne I: Functional maturation and proliferation of fetal pancreatic β-cells. Diabetes 40:89-93, 1991 (suppl 2)
26. Castells S, Mark E, Abaci F, et al: Growth retardation in fetal alcohol syndrome. Unresponsiveness to growth-promoting hormones. Dev Pharmacol Ther 3:232-241, 1981
27. Shepherd PR, Kahn BB: Glucose transporters and insulin action—Implications for insulin resistance and diabetes mellitus. N Engl J Med 341:248-257, 1999
28. Charron MJ, Katz EB, Olson AL: GLUT4 gene regulation and manipulation. J Biol Chem 274:3253-3256, 1999
29. Holcomb IN, Kabakoff RC, Chan B, et al: FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. EMBO J 19:4046-4055, 2000

30. Kim KH, Lee K, Moon YS, et al: A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J Biol Chem 276:11252-11256, 2001

31. Steppan CM, Bailey ST, Bhat S, et al: The hormone resistin links obesity to diabetes. Nature 409:307-312, 2001

32. Savage DB, Sewter CP, Klenk ES, et al: Resistin/fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-γ action in humans. Diabetes 50:2199-2202, 2001

33. Moore GB, Chapman H, Holder JC, et al: Differential regulation of adipocytokine mRNAs by rosiglitazone in db/db mice. Biochem Biophys Res Commun 286:735-741, 2001

34. Levy JR, Davenport B, Clore JN, et al: Lipid metabolism and resistin gene expression in insulin-resistant Fischer 344 rats. Am J Physiol 282:E626-E633, 2002

35. Stenger J, Bielajew C: Comparison of TOBEC-derived total body fat with fat pad weights. Physiol Behav 57:319-323, 1995

36. Newby FD, DiGirolamo M, Cotsonis GA, et al: Model of spontaneous obesity in aging male Wistar rats. Am J Physiol 259: R1117-1125, 1990

37. Friedman JM, Halaas JL: Leptin and the regulation of body weight in mammals. Nature 395:763-770, 1998

38. Jaquet D, Leger J, Levy-Marchal C, et al: Ontogeny of leptin in human fetuses and newborns: Effect of intrauterine growth retardation on serum leptin concentrations. J Clin Endocrinol Metab 83:1243-1246, 1998

39. Jaquet D, Gaboriau A, Czernichow P, et al: Relatively low serum leptin levels in adults born with intra-uterine growth retardation. Int J Obes Relat Metab Disord 25:491-495, 2001

40. Fisher SE, Karl PI: Maternal ethanol use and selective fetal malnutrition. Recent Dev Alcohol 6:277-289, 1988

41. Cornier MA, Gayles EC, Bessesen DH: The effects of chronic ethanol consumption on energy balance in rats. Metabolism 51:787-791, 2002

42. Shibley IA, Pennington SN: Metabolic and mitotic changes associated with the fetal alcohol syndrome. Alcohol Alcohol 32:423-434, 1997

43. Fisher SE, Atkinson M, Holtzman I, et al: Effect of ethanol upon placental uptake of amino acids. Prog Biochem Pharmacol 18:216-223, 1981

44. Lin GW: Fetal malnutrition: A possible cause of the fetal alcohol syndrome. Prog Biochem Pharmacol 18:115-121, 1981

45. Tanaka H, Suzuki N, Arima M: Hypoglycemia in the fetal alcohol syndrome in rat. Brain Dev 4:97-103, 1982

46. Snyder AK, Jiang F, Singh SP: Effect of ethanol on glucose utilization by cultured mammalian embryos. Alcohol Clin Exp Res 16:466-470, 1992

47. Singh SP, Snyder AK, Singh SK: Effects of ethanol ingestion on maternal and fetal glucose homeostasis. J Lab Clin Med 104:176-184, 1984

48. Boden G, Cheen X, DeSantis RA, et al: Ethanol inhibits insulin action on lipolysis and on insulin release in elderly men. Am J Physiol E197-E202, 1993

49. Xu D, Dhillon AS, Abelmann A, et al: Alcohol-related diols cause acute insulin resistance in vivo. Metabolism 47:1180-1186, 1998

50. Xu YY, Bhavani K, Wands JR, et al: Ethanol inhibits insulin receptor substrate-1 tyrosine phosphorylation and insulin-stimulated neuronal thread protein gene expression. Biochem J 310:125-132, 1995

51. Sasaki Y, Wands JR: Ethanol impairs insulin receptor substrate-1 mediated signal transduction during rat liver regeneration. Biochem Biophys Res Commun 199:403-409, 1994

52. Lillioja S, Mott DM, Howard BV, et al: Impaired glucose tolerance as a disorder of insulin action: Cross-sectional and longitudinal studies in Pima Indians. N Engl J Med 318:1217-1225, 1988

53. Warram JH, Martin BC, Krolewski AS, et al: Slow glucose removal rate and hyperinsulinemia precede the development of type 2 diabetes in the offspring of diabetic parents. Ann Intern Med 113:909-915, 1990