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# Prenatal cytokine exposure results in obesity and gender-specific programming

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**Dahlgren, Jovanna, Cecilia Nilsson, Eva Jennische, Hoi-Por Ho, Elias Eriksson, Aimon Niklasson, Per Björntorp, Kerstin Albertsson Wikland, and Agneta Holmäng.** Prenatal cytokine exposure results in obesity and gender-specific programming. *Am J Physiol Endocrinol Metab* 281: E326–E334, 2001.—Prenatal events appear to program hormonal homeostasis, contributing to the development of somatic disorders at an adult age. The aim of this study was to examine whether maternal exposure to cytokines or to dexamethasone (Dxm) would be followed by hormonal consequences in the offspring at adult age. Pregnant rats were injected on days 8, 10, and 12 of gestation with either human interleukin-6 (IL-6) or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or with Dxm. Control dams were injected with vehicle. All exposed offspring developed increased body weight ( $P < 0.05$ – $0.001$ ), apparently due to an increase of 30–40% in adipose tissue weight ( $P < 0.05$ – $0.01$ ). Corticosterone response to stress was increased in the IL-6 group ( $P < 0.05$ – $0.01$ ). Dxm-treated male rats exhibited blunted Dexamethasone suppression test results. In male rats, insulin sensitivity was decreased after IL-6 exposure ( $P < 0.01$ ), whereas basal insulin was elevated in the TNF- $\alpha$  group ( $P < 0.01$ ). In female rats, plasma testosterone levels were higher in all exposed groups compared with controls ( $P < 0.01$ – $0.001$ ), with the exception of Dxm-exposed offspring. Males in the TNF- $\alpha$  group showed decreased locomotor activity ( $P < 0.05$ ), and females in the IL-6 group showed increased locomotor activity ( $P < 0.05$ ). These results indicate that prenatal exposure to cytokines or Dxm leads to increased fat depots in both genders. In females, cytokine exposure was followed by a state of hyperandrogenicity. The results suggest that prenatal exposure to cytokines or Dxm can induce gender-specific programming of neuroendocrine regulation with consequences in adult life.

glucocorticoids; intrauterine exposure; hypothalamic-pituitary-adrenal axis; hypothalamic-pituitary-gonadal axis

SEVERAL STUDIES HAVE SUGGESTED that events that occur prenatally and early in life may play an important role in the pathogenesis of diseases at adult age in both animals (5, 29, 40) and humans (4, 40). Different kinds

of stressors during a defined developmental stage, or “window,” result in persisting changes in the behavioral (43) and hormonal (18, 26) responses to stress in adulthood, and in male rats may induce hormonal feminization, with low testosterone levels as well as feminine sexual behavior (15, 46).

There seem to be gender differences, although conflicting results have been reported in this regard (14, 15, 26). Examples include an altered stress response in females but not in males (26), decreased testosterone levels in males and elevated androstenedione in females (15), and both decreased adrenal weight and more pronounced histological abnormalities, such as cellular degeneration in the zona fasciculata, in female than in male rats (14).

Maternal infection during pregnancy represents one form of stressful event for the fetus. Lipopolysaccharides (LPSs) from gram-negative bacteria have been shown to induce permanent neuroendocrine changes (41) via the release of the cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and TNF- $\alpha$  (15, 21, 44). This has been found after prenatal (15) and neonatal exposure (41).

Damaged placentas may be permeable to cytokines (11), but little is known about the permeability for cytokines in a healthy placenta (8). In early pregnancy, the placenta appears to be permeable to infective agents (1). IL-6 receptors are widely expressed in human and murine tissues, such as the brain, spinal cord, adrenals, and the trophoblasts of the placenta. In humans, this is already found from 8 days postconception (10).

Cytokines, with the exception of IL-1 $\beta$ , are commonly believed not to penetrate the blood-brain barrier (BBB) but apparently reach the brain through other pathways, inducing the production of corticotropin-releasing hormone and prostaglandins (21, 23). Cytokines may also exert an influence at the levels of the adrenal (44) or the central nervous system by stimulating peripheral visceral vagal afferent nerves (21).

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Saturable transport of IL-6 and TNF- $\alpha$  crossing the BBB has previously been reported (3).

In humans, the amount of cortisol passing the placenta is limited, because the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase, or 11 $\beta$ -HSD, type 2, converts most cortisol to less active cortisone before it reaches the fetus (40). Dxm, however, a potent synthetic glucocorticoid without mineralocorticoid activity, passes the placenta without being inactivated and affects fetal growth (5, 40). Dxm exposure in late, but not early, pregnancy has been reported to result in reduced birth weight, hyperinsulinemia, increased hepatic glucocorticoid receptor (GR) expression (29), and hypertension at adult age (5), illustrating the apparent phenomenon of a "window" of time sensitivity of the fetus to such impacts.

In the present study, IL-6 and TNF- $\alpha$  were selected for further exploration in this regard. To avoid insensitive time periods of fetal response, repeated injections were given every 2nd day from *day 8* to *day 12* of gestation, which in rats is a period of early fetal brain development (30, 32). The effects of the cytokines were compared with those of exposure to Dxm to explore whether the effects are similar to those of exposure to glucocorticoids.

## METHODS

**Animals.** Timed-pregnant nulliparous Wistar rats (purchased from B&K Universal, Sollentuna, Sweden), weighing  $232 \pm 9$  g (range 189–298 g), were housed with one animal to a cage under controlled conditions with a temperature of  $21 \pm 1^\circ\text{C}$ , humidity of 55–65%, and lights on from 0700 to 1900. Animals received a commercial rat chow (containing 18.7% protein, 4.7% fat, and 63% carbohydrates with a sufficient supply of vitamins and minerals, purchased from B&K Universal) and tap water ad libitum. Pups were raised with a lactating mother until 4 wk of age. Thereafter, they lived in cages, with three animals from the same group per cage.

The study was approved by the Animal Ethics Committee of the University of Göteborg, Goteborg, Sweden.

**Dams and litters.** Dams were randomly divided into groups of three mothers each, with the exception of the control group, which consisted of four dams. Male offspring were also brought from five other Dxm-exposed dams and three more control dams. The dams were injected on *days 8, 10, and 12* of gestation, either intraperitoneally with 9  $\mu\text{g}/\text{kg}$  human IL-6 ( $n = 3$ ) or 4.5  $\mu\text{g}/\text{kg}$  TNF- $\alpha$  ( $n = 3$ ) (Boehringer Mannheim Biochemica, Mannheim, Germany), both dissolved in phosphate-buffered saline (PBS; Roche Diagnostics, Bromma, Sweden), or intramuscularly with 100  $\mu\text{g}/\text{kg}$  Dxm ( $n = 8$ ) (Merck Sharp & Dohme, Haarlem, The Netherlands). Control dams ( $n = 8$ ) were injected intraperitoneally with 0.8 ml of PBS. Blood samples from the mothers were collected 4 h after injection to determine levels of ACTH and corticosterone.

Gestation lasted for 21 days. There was no significant difference in the size of litters between the dams of exposed groups and the control group. The mean number of pups was  $8.7 \pm 1.9$  (range 5–11) for the IL-6 group,  $6.3 \pm 1.7$  pups (range 3–8) for the TNF- $\alpha$  group,  $8.6 \pm 0.6$  pups (range 7–11) for the Dxm-exposed group, and  $8.8 \pm 1.7$  (2–13) pups for the control group. At birth, pups were weighed and placed with their mothers. To equalize the number of pups per mother, they were redistributed after 1 wk so that the numbers of

pups within each experimental group were 9–12 (4–6 males and 5–6 females) per lactating mother.

Male and female offspring were brought up together. At 4 wk, the pups were weaned. Eight Dxm-exposed pups (4 male and 4 female) from one lactating mother died at *week 3* after delivery without any signs of infection. The total numbers of offspring were 12 females in the IL-6 group, 10 females in the TNF- $\alpha$  group, 8 females in the Dxm group, and 12 female controls. The male offspring numbers were 9 for the IL-6 group, 7 for the TNF- $\alpha$  group, 16 for the Dxm group, and 18 male controls.

**Food intake.** At 9–10 wk of age, food consumption for each cage (three animals per cage) was recorded every 2nd day for 8 days and calculated as food intake in grams per rat per day. The intake was measured on the following day by subtracting the uneaten food plus spillage from the total food given.

**Stress test procedure.** At 5 wk of age, the corticosterone stress response was tested between 0730 and 0900 by exposing the animals to novel-environment stress (25). After a 4-wk rest period without injections, tests, or any other manipulations except for the daily animal-keeping routine and weekly weight registration, the rats were singly brought from their home cages within the animal room to a novel environment (new cages, laboratory room, loud background, and bright light). For estimation of the basal plasma levels of corticosterone, great care was taken to keep the rats undisturbed the night before the experiment. A prestress blood sample (30  $\mu\text{l}$ ) was taken immediately before the animals were placed in the new cage, less than 25 s after removal from the home cage. Blood tail samples (30  $\mu\text{l}$ ) were then taken from the same animals 15, 30, 45, 60, 90, and 120 min after exposure to the novel environment.

**Dexamethasone suppression test.** At 6 wk, the Dexamethasone suppression test, modified according to Oxenkrug et al. (31), was performed in all females and a subgroup of males. Pilot tests with 7-wk-old Wistar control rats from another batch of rats had previously been performed to determine an optimal dose for partial suppression of endogenous corticosterone levels, which would enable us to distinguish individuals with resistant suppression from those with normal response. The dose (0.15  $\mu\text{g}$  Dxm/100 g body wt im) was decided on for this purpose. Tests were started randomly on nonfasting rats between 0900 and 1000. Tail blood was collected before the test for determination of basal levels of corticosterone, and then 2, 4, and 6 h after injection of Dxm.

**Vaginal smear.** Vaginal smears were obtained daily during 8–11 wk of age to determine the estrous cycle (42). A cycle is divided into four stages; estrus, diestrus 1, diestrus 2, and proestrus. The usual duration of a cycle in rats is  $\sim 4$  days. In this study, cycles of 4 or 5 days, with clear ovulation (measured as a characteristic, rich amount of epithelial cells without leukocytes in the smears), were considered normal.

**Baseline hormone levels.** At 8–11 wk of age, blood samples were collected from a nick in the tail, after fasting the rats overnight, to determine levels of glucose and insulin (both *weeks 8 and 10*) and leptin (*week 9*). In males, testosterone and progesterone levels were measured at 10 wk of age, whereas in females, testosterone, 17 $\beta$ -estradiol, and progesterone levels were analyzed at 11 wk of age, the day after estrus, in other words at the beginning of diestrus 1 (when these hormone levels are known to be still low compared with levels during the proestrus phase) (36). All samples were taken between 0800 and 0900 on the same day.

**Locomotion test.** Locomotor activity was assessed at 9 (males) and 10 (females) wk of age with photocell animal motility meters in eight soundproof, ventilated boxes connected to a computer (Kungsbacka mät-och regler teknik,

Fjärås, Sweden). The activity boxes, with a floor area of 700 × 700 mm, were equipped with two rows of eight photocells each. Locomotor activity during 60 min was registered, in dim light, as the breaking of a sequence of beams, representing movement in a single direction (12). The order in which the rats were placed in the boxes was randomized. The time of day (between 1200 and 1500) and time of feeding were standardized for all groups.

**Euglycemic hyperinsulinemic clamp.** Rats were subjected to a euglycemic hyperinsulinemic clamp, as described previously (20), at 10–11 wk of age in males and 12–13 wk of age in females. The animals were anesthetized with 125 mg/kg body weight of thiobutabarbital sodium (Inactin, RBI, Natick, MA). Thereafter, catheters were inserted into the left carotid artery for blood sampling and into the right jugular vein for infusion of glucose and insulin. The body temperature was maintained at 37°C with a heating blanket. After a bolus injection, insulin (Human Actrapid, 100 U/ml, Novo, Copenhagen, Denmark) was continuously infused at a rate of 8 mU·kg<sup>-1</sup>·min<sup>-1</sup>. A 15% glucose solution in physiological saline was administered to maintain the plasma glucose concentration at 7 mM. Glucose was infused at a rate guided by glucose concentration measurements in 30 µl of blood at regular intervals (every 5 min during the first 40 min then every 10 min). Steady state for plasma glucose and insulin concentrations was reached at ~50 min and was maintained to the end of clamp, in this study until 170 min. At 0, 40, 80, 120, and 160 min of infusion, 250-µl blood samples were taken for determination of the insulin concentration. Erythrocytes were given back continuously during sample collection. A total of <2 ml of blood was used for the measurements and was compensated for by the infusion volumes. Two male rats (one control and one TNF-α rat) and four female rats (one control, one IL-6, and two TNF-α rats) died during the clamp procedure.

**Tissues.** At the completion of the clamp, the rats were decapitated. The adrenals, thymus, heart, spleen, gonads, and extensor digitorum longus, tibialis anterior, and soleus muscles of the hindlimb, as well as the epididymal, mesenteric, and retroperitoneal adipose tissues, were dissected out and weighed immediately.

**Histological examination.** After the ovaries and adrenals were weighed, they were fixed in 4% buffered formaldehyde. They were then rinsed in PBS containing 7.5% sucrose and frozen in liquid nitrogen. Cryostat sections, 5 µm thick, were prepared and stained with hematoxylin and eosin, dehydrated, and mounted.

**Analytical methods.** Blood was collected in heparinized microtubes and centrifuged immediately in a microcentrifuge at 4°C. Blood for ACTH determination in the pregnant mothers was collected in chilled EDTA microtubes and centrifuged. Plasma glucose was determined enzymatically in 15-µl samples on a YSI 2700 SELECT biochemical analyzer (Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin was analyzed with a rat insulin RIA kit (Linco Research, St. Charles, MO) and human insulin, administered during the clamp, with a double-antibody radioimmunoassay (Pharmacia, Uppsala, Sweden). Testosterone was measured with a solid-phase radioimmunoassay (Coat-A-Count Total Testosterone, Diagnostic Products, Los Angeles, CA). Both 17β-estradiol and progesterone were assayed with commercially available enzyme immunoassays (ELISA; Biomar Diagnostic Systems, Marburg, Germany). ACTH levels were determined with an immunoradiometric assay (IDS, Boldon, UK) and corticosterone by a radioimmunoassay (RSL <sup>125</sup>I corticosterone RIA; ICN Biomedicals, Costa Mesa, CA), with a detection level above 25 ng/ml. Leptin was determined by a radioimmunoassay (rat leptin RIA kit); Linco Research, St. Louis, MO.

**Statistical methods.** Distribution of variables is given as means ± SE. A Mann-Whitney nonparametric *U*-test was used for a comparison between the treatment groups and the control group. A Wilcoxon signed-rank test was performed for paired comparison between basal corticosterone and response to the Dexamethasone suppression test. A two-way ANOVA was performed for comparison of locomotion activity between treatment and control groups. A two-tailed *P* value of <0.05 was considered statistically significant. A χ<sup>2</sup>-test was performed to compare statistical differences between vaginal smears in treated animals and controls.

## RESULTS

**Dams and litters.** TNF-α-injected dams showed significantly elevated corticosterone levels 4 h after the injection (i.e., on days 8 and 10 of gestation) compared with controls (TNF-α dams had mean corticosterone levels of 640 ± 45 ng/ml, *P* < 0.05; IL-6-treated dams, of 592 ± 51 ng/ml, *P* = 0.09; and controls, of 432 ± 50 ng/ml). IL-6-injected dams showed significantly elevated ACTH levels 4 h postinjection (IL-6 dams had levels of 194 ± 24 pmol/l, *P* < 0.05; the TNF-α group, of 156 ± 24 pmol/l, *P* = 0.16; and controls, of 99 ± 26

Table 1. Development of total body weight from 3 to 10 wk of age in males, and from 3 to 12 wk of age in females, in control, IL-6, TNF-α, and Dxm groups

Group	<i>n</i>	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
Control											
Male	17	35 ± 2	64 ± 2	103 ± 3	147 ± 5	192 ± 6	228 ± 3	254 ± 6	317 ± 3		
Female	12	31 ± 1	58 ± 1	89 ± 3	119 ± 3	146 ± 3	163 ± 3	176 ± 3	189 ± 4	204 ± 6	229 ± 2
IL-6											
Male	9	43 ± 1†	79 ± 1‡	118 ± 2†	165 ± 3*	208 ± 4	255 ± 4‡	277 ± 5*	345 ± 6†		
Female	12	42 ± 1‡	76 ± 1‡	107 ± 2‡	138 ± 2‡	160 ± 3†	175 ± 3*	187 ± 3	199 ± 4	206 ± 3	237 ± 5
TNF-α											
Male	7	35 ± 4	71 ± 6	109 ± 8	153 ± 11	201 ± 12	246 ± 13	265 ± 14	346 ± 11†		
Female	10	36 ± 3	70 ± 4†	104 ± 4†	138 ± 4†	165 ± 6*	181 ± 6*	192 ± 6	205 ± 8	211 ± 7	238 ± 6
Dxm											
Male	15	48 ± 2‡	81 ± 2‡	118 ± 4†	165 ± 5*	214 ± 4†	238 ± 6	271 ± 7	324 ± 5		
Female	8	41 ± 2	75 ± 4‡	107 ± 4‡	138 ± 4‡	162 ± 5†	177 ± 5†	188 ± 6*	200 ± 5*	205 ± 6	232 ± 5

Values are means ± SE (*n* = no./group) expressed in g. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; Dxm, dexamethasone. \**P* < 0.05, †*P* < 0.01, ‡*P* < 0.001 vs. control rats (Mann-Whitney nonparametric *U*-test).

Table 2. Total body weight and weights of epididymal, parametrial, retroperitoneal, and mesenteric adipose tissue in wk 12 (females) and wk 10 (males) in control, IL-6, TNF- $\alpha$ , and Dxm groups

Group	n	Total body weight, g	Adipose Tissue, g/kg body wt			
			Epididymal	Parametrial	Retroperitoneal	Mesenteric
Control						
Males	17	317 $\pm$ 3	14.2 $\pm$ 0.8		12.1 $\pm$ 0.7	8.1 $\pm$ 0.5
Females	12	229 $\pm$ 3		21.0 $\pm$ 1.0	11.8 $\pm$ 0.8	11.4 $\pm$ 0.4
IL-6						
Males	9	345 $\pm$ 6 $\dagger$	17.8 $\pm$ 1.0 $\dagger$		16.1 $\pm$ 1.4 $\dagger$	10.5 $\pm$ 1.1*
Females	12	237 $\pm$ 5		28.1 $\pm$ 1.4 $\dagger$	17.1 $\pm$ 1.1 $\dagger$	12.3 $\pm$ 0.7
TNF- $\alpha$						
Males	7	346 $\pm$ 11 $\dagger$	17.0 $\pm$ 0.4		16.0 $\pm$ 0.4 $\dagger$	9.4 $\pm$ 0.5
Females	10	238 $\pm$ 6		26.2 $\pm$ 1.9*	16.2 $\pm$ 1.1 $\dagger$	10.0 $\pm$ 0.9
Dxm						
Males	15	324 $\pm$ 5	16.5 $\pm$ 1.0		15.3 $\pm$ 0.9 $\dagger$	9.0 $\pm$ 0.4
Females	15	232 $\pm$ 5		29.5 $\pm$ 1.9 $\dagger$	18.6 $\pm$ 1.6 $\dagger$	12.7 $\pm$ 0.7

Values are means  $\pm$  SE; n, no./group. \* $P$  < 0.05,  $\dagger P$  < 0.01 vs. control rats (Mann-Whitney  $U$ -test).

pmol/l). Dxm-injected dams showed suppressed corticosterone and ACTH secretion (below detection levels).

There were no differences between the body weight of newborn pups in the cytokine- and Dxm-injected groups and the control group (IL-6 rats had a body weight of 6.5  $\pm$  0.1 g; TNF- $\alpha$  rats, of 6.6  $\pm$  0.1 g; Dxm-exposed rats, of 6.6  $\pm$  0.1 g; and controls, of 6.6  $\pm$  0.1 g).

**Body weight development and body composition.** Table 1 shows total body weight development for male (3–10 wk of age) and female rats (3–12 wk of age). Until 8 wk of age, prenatal exposure to cytokines and Dxm resulted in significantly elevated body weight ( $P$  < 0.05–0.001) in both genders and all treated groups except for the TNF- $\alpha$  males.

Table 2 shows total body and tissue weights at decapitation for males and females. The IL-6 and TNF- $\alpha$  males had an elevated total body weight at decapitation ( $P$  < 0.01). In all treated rats, males as well as females, retroperitoneal fat depots were significantly heavier ( $P$  < 0.01). In males, epididymal and mesenteric fat depots were only significantly larger in IL-6-treated animals ( $P$  < 0.01 and  $P$  < 0.05, respectively) compared with controls. In contrast, in all treated

females, parametrial fat depots, but not mesenteric fat depots, were significantly increased ( $P$  < 0.05–0.01).

No statistically significant differences were found in the weights of the extensor digitorum longus, tibialis anterior, and soleus muscles except in Dxm females, in which the soleus muscle weighed significantly less (0.68  $\pm$  0.02 g) than in controls (0.75  $\pm$  0.02 g,  $P$  < 0.01). The weights of the adrenals, thymus, gonads, and heart were not different between the groups (data not shown).

**Food intake.** At 9–10 wk of age, the average food intake for male rats was 24.8  $\pm$  1.3 g per rat and day for the IL-6 group, 21.5  $\pm$  1.2 g for TNF- $\alpha$ -exposed rats, 25.2  $\pm$  0.9 g for the Dxm group, and 25.0  $\pm$  0.5 g for the controls. Female rats showed an average food intake at 9–10 wk of age of 18.8  $\pm$  3.8 g for the IL-6 group, 20.4  $\pm$  0.9 g for the TNF- $\alpha$  group, 20.2  $\pm$  0.9 g for Dxm-exposed rats, and 21.0  $\pm$  0.8 g for controls. None of these differences was significant compared with controls.

**Stress-induced corticosterone secretion.** Table 3 shows the basal plasma corticosterone and plasma corticosterone response to the novel-environment stress test, expressed as the difference between plasma

Table 3. Basal plasma corticosterone and plasma corticosterone response to novel-environment stress test, expressed as the difference between plasma corticosterone values at 15, 30, 45, 60, 90, and 120 min and basal plasma corticosterone at 5 wk of age in control, IL-6, TNF- $\alpha$ , and Dxm groups

Group	n	Basal, 0 min	15 min – 0 min	30 min – 0 min	45 min – 0 min	60 min – 0 min	90 min – 0 min	120 min – 0 min
Control								
Males	17	242 $\pm$ 26	152 $\pm$ 31	214 $\pm$ 30	168 $\pm$ 25	148 $\pm$ 30	114 $\pm$ 31	102 $\pm$ 32
Females	12	364 $\pm$ 54	151 $\pm$ 68	242 $\pm$ 74	183 $\pm$ 80	222 $\pm$ 39	99 $\pm$ 78	116 $\pm$ 86
IL-6								
Males	9	218 $\pm$ 40	219 $\pm$ 80	260 $\pm$ 66	310 $\pm$ 55*	315 $\pm$ 48 $\dagger$	264 $\pm$ 55*	246 $\pm$ 57*
Females	12	296 $\pm$ 55	394 $\pm$ 53 $\dagger$	356 $\pm$ 52	294 $\pm$ 56	218 $\pm$ 78	248 $\pm$ 58	133 $\pm$ 59
TNF- $\alpha$								
Males	7	340 $\pm$ 80	200 $\pm$ 86	238 $\pm$ 93	211 $\pm$ 75	141 $\pm$ 71	82 $\pm$ 81	93 $\pm$ 68
Females	10	470 $\pm$ 18	139 $\pm$ 44	129 $\pm$ 60	139 $\pm$ 58	136 $\pm$ 49	17 $\pm$ 45	–32 $\pm$ 50
Dxm								
Males	15	177 $\pm$ 34	181 $\pm$ 40	202 $\pm$ 41	181 $\pm$ 42	149 $\pm$ 46	156 $\pm$ 70	63 $\pm$ 42
Females	8	332 $\pm$ 51	268 $\pm$ 41	308 $\pm$ 44	248 $\pm$ 64	220 $\pm$ 78	150 $\pm$ 58	46 $\pm$ 51

Corticosterone values are means  $\pm$  SE expressed in ng/ml. \* $P$  < 0.05;  $\dagger P$  < 0.01 vs. control rats (Mann-Whitney  $U$ -test).

Table 4. Basal plasma corticosterone and plasma corticosterone response to the Dexamethasone suppression test expressed as the difference between plasma corticosterone values at 2, 4, and 6 h after the test and the basal plasma corticosterone in control, IL-6, TNF- $\alpha$ , and Dxm groups

Group	n	Basal (0 min)	2 h - Basal 0 min	4 h - Basal 0 min	6 h - Basal 0 min
Control					
Males	12	152 $\pm$ 31	1 $\pm$ 36	-96 $\pm$ 38§	-58 $\pm$ 41
Females	12	300 $\pm$ 55	-77 $\pm$ 56	96 $\pm$ 84	421 $\pm$ 79§
IL-6					
Males	9	186 $\pm$ 32	9 $\pm$ 39	-113 $\pm$ 38§	-2 $\pm$ 50
Females	12	388 $\pm$ 61	-126 $\pm$ 66	-54 $\pm$ 61	124 $\pm$ 128
TNF- $\alpha$					
Males	7	376 $\pm$ 37†	-132 $\pm$ 57	-193 $\pm$ 40§	-204 $\pm$ 68§
Females	10	463 $\pm$ 55*	-66 $\pm$ 45	71 $\pm$ 44	143 $\pm$ 51§
Dxm					
Males	5	234 $\pm$ 59	-7 $\pm$ 37	11 $\pm$ 18	54 $\pm$ 62
Females	8	373 $\pm$ 54	-100 $\pm$ 41	-73 $\pm$ 40	113 $\pm$ 74

Values are means  $\pm$  SE expressed in ng/ml. \* $P$  < 0.05; † $P$  < 0.01 vs. control rats (Mann-Whitney nonparametric  $U$ -test). § $P$  < 0.05, changes from basal levels within each group (Wilcoxon signed-rank Test, for paired test).

corticosterone values at 15, 30, 45, 60, 90, and 120 min and the basal plasma corticosterone at 5 wk of age.

In males, the basal plasma corticosterone levels did not differ between the groups. IL-6-exposed rats showed a significantly higher stress response than did control rats during 45–120 min after stress exposure ( $P$  < 0.05–0.01). In females, the IL-6 group showed at 15 min a larger increase in corticosterone levels than did control rats ( $P$  < 0.01).

In both genders, the stress response in the TNF- $\alpha$  and Dxm groups did not differ compared with the control group.

**Dexamethasone suppression test.** Table 4 shows the basal plasma corticosterone and plasma corticosterone response to Dexamethasone suppression expressed as the difference between plasma corticosterone values at 2, 4, and 6 h after the Dxm injection and the basal plasma corticosterone level.

In male rats, the TNF- $\alpha$  group showed elevated basal corticosterone levels ( $P$  < 0.01), but at 4 h, corticosterone levels (compared to basal values) were significantly suppressed in all groups ( $P$  < 0.05) except for the Dxm rats. The TNF- $\alpha$  group showed suppression also at 6 h after Dxm injection ( $P$  < 0.05).

In female rats, the TNF- $\alpha$  group showed elevated basal corticosterone levels ( $P$  < 0.05). At 6 h, the TNF- $\alpha$  and control groups had significantly elevated values compared with basal values ( $P$  < 0.05).

**Baseline hormone levels.** Table 5 shows fasting plasma concentrations of insulin, glucose, leptin, and testosterone in male and female rats.

Compared with male controls, fasting insulin levels in TNF- $\alpha$ -exposed males were significantly elevated ( $P$  < 0.01), whereas glucose levels showed no statistically significant difference. There was a tendency for leptin to be higher and for testosterone to be lower in all treated males, but this difference did not reach statistical significance.

In female rats, the IL-6 ( $P$  < 0.01) and Dxm ( $P$  < 0.05) groups exhibited elevated leptin levels. Testosterone levels were elevated in the IL-6 ( $P$  < 0.001) and TNF- $\alpha$  female groups ( $P$  < 0.01) but not in the Dxm group. Fasting plasma insulin and glucose levels showed no significant differences between the treated groups and the controls.

No differences were found in the 17 $\beta$ -estradiol and progesterone levels (data not shown).

Table 5. Fasting plasma levels of leptin at week 9, testosterone at weeks 10–11, and insulin and glucose at week 9 in control, IL-6, TNF- $\alpha$ , and Dxm groups

Group	n	Leptin, ng/ml	Testosterone, nmol/l	Insulin, mU/L	Glucose, mmol/l
Control					
Male	17	1.0 $\pm$ 0.08	4.6 $\pm$ 0.84	6.6 $\pm$ 0.72	3.4 $\pm$ 0.20
Female	12	1.3 $\pm$ 0.1	0.15 $\pm$ 0.01	8.1 $\pm$ 1.0	4.1 $\pm$ 0.2
IL-6					
Male	9	1.5 $\pm$ 0.8	3.2 $\pm$ 0.56	9.6 $\pm$ 1.7	3.8 $\pm$ 0.38
Female	12	2.0 $\pm$ 0.2†	0.27 $\pm$ 0.02‡	7.0 $\pm$ 0.7	4.8 $\pm$ 0.2
TNF- $\alpha$					
Male	7	2.0 $\pm$ 0.57	3.9 $\pm$ 0.98	14.7 $\pm$ 3.2†	4.3 $\pm$ 0.68
Female	10	1.4 $\pm$ 0.1	0.23 $\pm$ 0.02†	9.5 $\pm$ 1.3	4.5 $\pm$ 0.2
Dxm					
Male	15	1.4 $\pm$ 0.77	2.6 $\pm$ 0.90	10.0 $\pm$ 3.0	3.5 $\pm$ 0.19
Female	8	2.8 $\pm$ 0.5*	0.16 $\pm$ 0.01	8.2 $\pm$ 1.6	4.7 $\pm$ 0.3

Values are means  $\pm$  SE. \* $P$  < 0.05, † $P$  < 0.01, ‡ $P$  < 0.001 vs. control rats (Mann-Whitney nonparametric  $U$ -test).

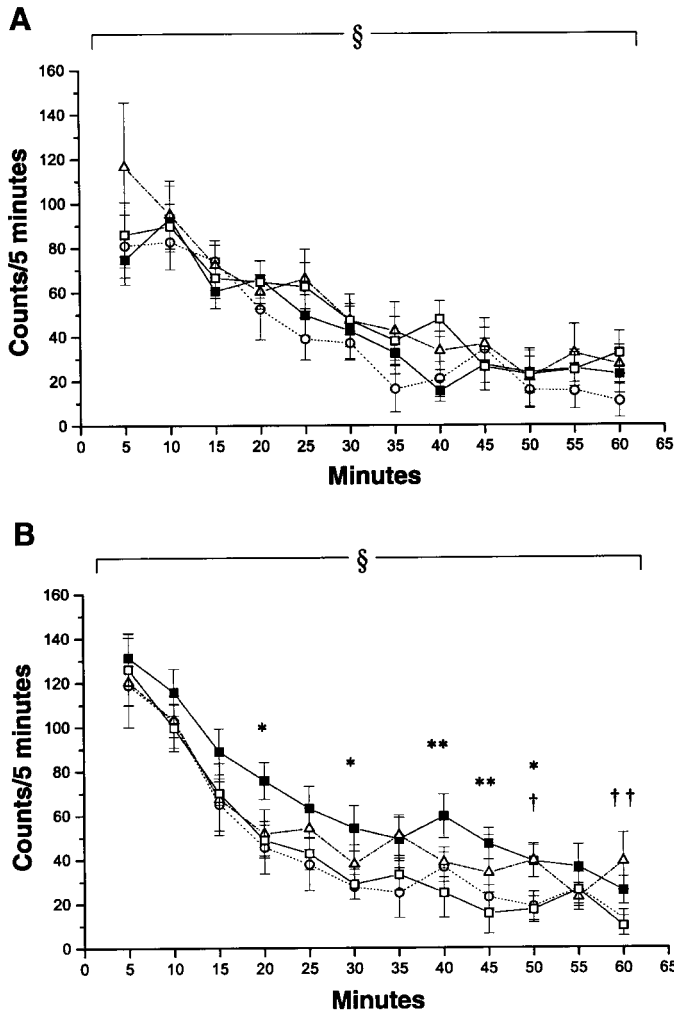


Fig. 1. A: locomotor activity (counts/5 min) in male rats in the interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), dexamethasone (Dxm), and control groups. ■, IL-6-exposed rats ( $n = 12$ ); ○, TNF- $\alpha$ -exposed rats ( $n = 10$ );  $\Delta$ , Dxm-exposed rats ( $n = 8$ ); □, controls ( $n = 12$ ). Experimental conditions are presented in METHODS. § $P < 0.05$  for the TNF- $\alpha$  group compared with control rats, measuring mean activity during 0–60 min. Statistical analysis is by 2-way ANOVA. Data are means  $\pm$  SE. B: locomotor activity (counts/5 min) in female rats in the IL-6, TNF- $\alpha$ , Dxm, and control groups. \* $P < 0.05$ , \*\* $P < 0.01$  for IL-6; † $P < 0.05$  and †† $P < 0.01$  for the Dxm group compared with control group. § $P < 0.05$  for IL-6 group, measuring mean activity during 0–60 min. Analysis by 2-way ANOVA. Data are means  $\pm$  SE.

**Locomotion test.** ANOVA results for all groups indicate that the exploratory locomotion was dependent on time ( $P < 0.001$ ).

Figure 1A shows locomotor activity (counts/5 min) in male rats. The TNF- $\alpha$  group displayed less motor activity ( $P < 0.05$ ) throughout the investigated time period (60 min) than did the controls.

Figure 1B shows locomotor activity (counts/5 min) in female rats. The IL-6 group exhibited a higher activity rate than did the control group at 20, 30, 40, 45, and 50 min ( $P < 0.05$ – $< 0.01$ ), and so also did the Dxm group at 50 ( $P < 0.05$ ) and 60 min ( $P < 0.01$ ), but when the mean activity rate was calculated for the entire mea-

sured 60 min, only the IL-6 group showed a higher activity rate ( $P < 0.05$ ).

**Euglycemic hyperinsulinemic clamp.** Figure 2 depicts the glucose infusion rate for each individual during the euglycemic hyperinsulinemic clamp in male rats. IL-6-treated rats showed a significantly lower glucose uptake ( $18.9 \pm 1.1 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ;  $P < 0.01$ ) during steady state (50–170 min) than did control rats ( $22.9 \pm 0.8 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). The glucose infusion rate in the TNF- $\alpha$  ( $20.7 \pm 1.2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) and Dxm ( $22.1 \pm 0.9 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) male rats did not differ from that of the control group. During steady state, neither plasma insulin (levels in the IL-6 group were  $190 \pm 10 \text{ mU/l}$ ; in the TNF- $\alpha$  group,  $217 \pm 30 \text{ mU/l}$ ; in the Dxm group,  $211 \pm 5.1 \text{ mU/l}$ ; and in controls,  $194 \pm 15 \text{ mU/l}$ ) nor plasma glucose (levels in the IL-6 group were  $6.9 \pm 0.0 \text{ mmol/l}$ ; in the TNF- $\alpha$  group,  $6.8 \pm 0.1 \text{ mmol/l}$ ; in the Dxm group,  $6.9 \pm 0.1 \text{ mmol/l}$ ; and in the controls,  $6.9 \pm 0.1 \text{ mmol/l}$ ) differed among the groups.

There were no significant differences in the glucose infusion rate between the treated females and control females (data not shown).

**Vaginal smear.** Estrous cycles were counted during a period of 18 days (4 completed cycles). Normal estrous cycles were found in 50, 75, 75, and 80% of TNF- $\alpha$ , IL-6, Dxm, and control females, respectively. These differences were not statistically different when analyzed with the  $\chi^2$ -test.

**Histological examination.** The ovaries of the cytokine and Dxm-treated offspring showed a normal histology. They had a similar relative proportion of stroma and gamete-producing structures in the cortex to that seen in ovaries from control animals. Furthermore, all stages of developing follicles, as well as corpora lutea, could be identified.

**DISCUSSION**

In both humans and rats, perinatal exposure to various factors has been shown to be followed by permanent, disease-generating consequences during adulthood. LPSs (35, 41), cytokines (15), and glucocorticoids (5, 14, 29) are believed to be involved, programming the

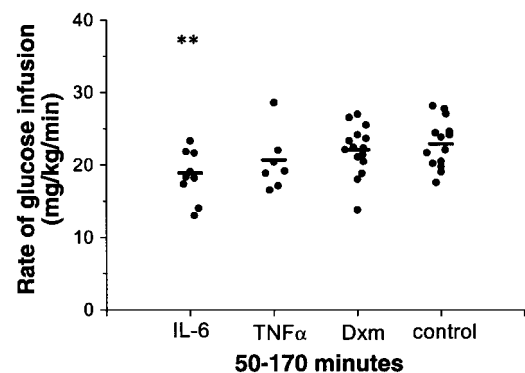


Fig. 2. Rate of glucose infusion in male rats at steady state (50–170 min) during the euglycemic hyperinsulinemic clamp ( $8 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). Individual values are shown, with means given as lines. \*\* $P < 0.01$  for IL-6 group compared with control rats. Data are means  $\pm$  SE (Mann-Whitney nonparametric  $U$ -test).

regulation of both the neuroendocrine and the autonomic nervous systems. The present study was therefore performed to focus on the metabolic and endocrine effects of cytokines on prenatally exposed offspring at adult age. The aim was also to try to sort out whether or not the effects are related to glucocorticoids, because both LPSs and cytokines are powerful stimulators of glucocorticoid secretion (35, 44).

In this study, multiple injections were used to cover the sensitive period when brain development is most pronounced in the rat fetus (32). The doses chosen were in accord with those used on adult rats in previous studies (6, 16, 19, 29, 38). The synthetic glucocorticoid Dxm was used to separate out the effects of glucocorticoids, because it is known to pass the placental barrier (29). Administration of Dxm during pregnancy will therefore expose the fetus to a powerful glucocorticoid.

The injection of pregnant dams with cytokines caused either elevated ACTH or corticosterone levels. The injection of Dxm, on the other hand, caused a significant inhibition of both ACTH and corticosterone secretion, which was expected because of feedback regulation (9).

**Obesity, food intake, and locomotor activity.** In this study, exposed offspring showed no body weight differences at birth; however, enlarged abdominal fat depots were found in both male and female adult rats. Leptin levels were significantly elevated in males from groups exposed to IL-6 and Dxm, in parallel with the increased amount of the abdominal fat depot. In all exposed females, leptin levels were 40–100% higher than in controls, although this difference did not reach statistical significance. Leptin, the adipocyte-derived hormone encoded by the obese gene (47), is an important regulator of energy balance through the various effects it has on food intake and thermogenesis. Leptin inhibits the activation of the hypothalamic-pituitary-adrenal (HPA) axis at hypothalamic (2, 17) and peripheral levels (7). Endotoxin and cytokines are known to increase plasma leptin (13, 24, 39). Interestingly, in a previous study, male rats exposed to early gestational undernutrition also developed obesity (22).

The elevated fat mass indicates a positive energy balance, in other words, an increased energy intake and/or decreased physical activity. Another explanation could be a decreased thermogenesis, which was not measured.

No measurable differences in food intake were seen in the male or female groups. Only the TNF- $\alpha$ -exposed male rats exhibited less locomotor activity, but this was not found in the IL-6- and Dxm-exposed males, and furthermore, the IL-6 and Dxm-treated female offspring showed increased physical activity. There is, therefore, no clear explanation for the increased body fat mass. In the face of the elevated leptin levels, it seems likely that an elevated energy intake may have been responsible, although this was not possible to discover with the method used.

**Corticosterone levels and stress sensitivity.** Perturbation in the regulation of the HPA axis was found in the IL-6 group, because they reacted to novel-environment

stress with an elevated response of corticosterone and showed a suppression of corticosterone secretion by Dxm. The basal corticosterone levels were elevated in the TNF- $\alpha$  groups, with normal elevated corticosterone response to the novel-environment and normal Dexamethasone suppression conditions compared with basal levels. This indicates an increased central facilitation of the activity of the HPA axis, with a remaining feedback control (9). There was no suppression by Dxm in the prenatally Dxm-exposed male rats. This finding suggests a blunted regulation of the HPA axis, with a diminished feedback control. Such changes in the regulation of the HPA axis are usually seen after repeated, severe challenges in adulthood, resulting in a burnout of the HPA axis (27). The blunted response to the Dexamethasone suppression may have been due to interference with the function of central GRs.

It therefore seems likely that, in the present study, Dxm exerted more powerful effects on the regulation of the HPA axis than did the cytokines, which induced endogenous hypersecretion of ACTH and corticosterone in the dams. This assumption appears to find support in the total suppression of corticosterone secretion in Dxm-exposed dams. Another putative explanation for the differences may be the direct effect of cytokines on the fetus, as they may alter the permeability of the placenta (11), interfering with the sensitivity or density receptors for glucocorticoids, testosterone, or interleukins (28).

All female rats showed higher basal corticosterone compared with males. Female corticosterone levels were also difficult to suppress with the same low Dxm dose used in males. This could be due to gender differences in corticotropin-releasing hormone (CRH) secretion, as estrogens are known to stimulate CRH gene expression (45).

**Androgenization of female rats.** There were consistently elevated testosterone values in the cytokine-treated females, with an increase of up to 80%. This is in accordance with previously reported findings of increased androstenedione in female rats after perinatal application of IL-1 $\beta$  (15). The tendency to more abnormal estrous cycles in the rats treated with TNF- $\alpha$  indicates that the hyperandrogenicity may be of ovarian origin, although no abnormalities were discovered in either weight or the histological appearance of ovaries, such as polycystic changes. TNF- $\alpha$  has been re-

Table 6. Summary of comparative results between IL-6, TNF- $\alpha$ , and Dxm groups and control groups

	Males			Female		
	IL-6	TNF- $\alpha$	Dxm	IL-6	TNF- $\alpha$	Dxm
Body weight	↑	↑	⇒	⇒	⇒	⇒
Adipose tissues	↑	↑	↑	↑	↑	↑
Stress response	↑	⇒	⇒	↑	⇒	⇒
Insulin sensitivity	↓	⇒	⇒	⇒	⇒	⇒
Fasting insulin	⇒	↑	⇒	⇒	⇒	⇒
Plasma leptin	⇒	⇒	⇒	↑	⇒	↑
Plasma testosterone	⇒	⇒	⇒	↑	↑	⇒
Locomotion activity	⇒	↓	⇒	↑	⇒	↑

ported to interfere with ovarian steroid production, suggesting follicular atresia (37), although this has not been studied after prenatal exposure.

Cytokines have also been found to interfere with the regulation of the hypothalamic-pituitary-gonadal axis (36). Cytokines of central origin alter the activity of the luteinizing hormone-releasing hormone-secreting neurons and inhibit luteinizing hormone secretion as well as ovulation. Peripheral cytokines, by contrast, interfere with sex steroid release at the gonadal level (19). It is therefore possible that the elevated testosterone levels in females may originate from such perturbations.

**Insulin resistance.** Male IL-6 rats showed insulin resistance in the clamp measurements, and the TNF- $\alpha$  group had elevated basal insulin values. This may have been due to the increased body fat mass and/or elevated corticosterone secretion during stress, both well established generators of such perturbations. In females there were no indications of insulin resistance despite enlarged fat depots and the tendency to an increased stress response of corticosterone. The reason for this gender difference is not known.

To summarize, all prenatally exposed offspring developed obesity at adult age. Cytokine exposure hypersensitized the HPA axis in both genders, whereas Dxm-exposed males showed blunted Dxm suppression. IL-6 males, but not IL-6 females, were found to have insulin resistance. Females were hyperandrogenized. To facilitate the overview of results in females and males, a summary is provided (Table 6).

It has been suggested that in humans, as in rats, the prenatal environment is of importance for the development of disease in adulthood. Retrospective observations, such as the epidemiological study of the impact of the Dutch famine on exposed offspring, have emphasized the importance of specific windows of sensitivity to induce permanent changes to hormonal programming. Exposure to famine during early gestation did not affect birth size but instead correlated with obesity in adulthood. This was more pronounced in women than in men (34). Exposure to famine during mid-trimester and late gestation resulted in low birth weight and insulin resistance (33), as well as other features of the metabolic syndrome (4).

The observations on rats reported in this work have similarities with those reported on humans, because HPA axis sensitization, obesity, and insulin resistance were found in exposed male rats. In addition to obesity, cytokine-exposed females were found to be hyperandrogenic. It seems likely that these abnormalities were due to a prenatal programming of the neuroendocrine axes. This study is an attempt to explore some of the mechanisms behind such programming. Cytokines may well be one piece in the multivariable puzzle of neonatal programming and risk of developing disease at adult age. To further elucidate the mechanisms involved, additional studies will be required.

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