OBESITY IS ONE OF THE MAJOR HEALTH ISSUES that has worldwide prevalence (1). In addition to genetic and lifestyle factors, growing evidence suggests that prenatal conditions could also contribute to the development of obesity in the offspring. According to Barker’s hypothesis, compromised in utero conditions can induce permanent alterations in tissue structure and function of the developing fetus and increase their susceptibility to diseases like obesity and diabetes in adult life (2, 17).

Several studies have shown that offspring of dams placed on low-protein diets or subjected to caloric restriction have low birth weight. These offspring subsequently undergo catchup growth that places them at higher risk for obesity, hypertension, and diabetes in adulthood (18, 24, 32). Apart from nutritional challenges, maternal stress that increases circulating glucocorticoids during pregnancy is also known to program the offspring for metabolic diseases (12, 37, 38). In humans, prenatal stress in the form of maternal bereavement or natural disasters during pregnancy has been shown to increase the risk for obesity in childhood (6, 27). Numerous studies in rodents and primates suggest that prenatal stress or dexamethasone administration reduces birth weight and predisposes the offspring to glucose intolerance, insulin resistance, and reduced β-cell mass, especially when they are exposed to a high-fat (HF) diet (7, 29, 33, 42). Prenatal stress also alters the expression of lipogenic and gluconeogenic enzymes in adipose tissue and liver that could lead to the development of obesity (7, 10, 42).

Although there is ample evidence in the literature supporting the metabolic programming effects of prenatal stress in both humans and animals, it remains unclear whether these effects could be exacerbated in individuals genetically predisposed to obesity. Considering the fact that in the US around 29% of women of reproductive age (20–39 yr) are obese (19), it is important to investigate whether maternal stress would introduce metabolic changes in the offspring of obese mothers. Therefore, we investigated the metabolic effects of prenatal stress in diet-induced obese (DIO) rats, a polygenic obese animal model developed from a Sprague-Dawley background by Levin et al. (26). These animals were subjected to repeated selective breeding after they were placed on a HF diet and had their tendency to gain weight observed. DIO rats resemble human obesity in their tendency to gain weight on HF diet exposure, and they also develop other metabolic alterations similar to obese humans. Their lean counterparts, the dietary resistant (DR) rats, do not gain weight after HF diet exposure and were used for comparison.

The mechanism by which prenatal stress leads to the development of obesity is not clear. One of the important contributing factors to obesity is hyperinsulinemia, and there is a strong likelihood that prenatal stress may promote hyperinsulinemia in the offspring (3). Hyperinsulinemia may be caused by overproduction of insulin by β-cells of the pancreas or by reduced insulin clearance from the body (22). Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a glycoprotein that promotes insulin clearance through receptor-mediated endocytosis in hepatocytes (14). Reduced expression of CEACAM1 causes hyperinsulinemia that is central to the development of metabolic disorders resulting from impairment of insulin clearance (8, 35). Therefore, we investigated...
the role of CEACAM1 in the development of obesity in prenatally stressed DIO and DR rats.

MATERIALS AND METHODS

Animals and treatment. Adult male and female DIO and DR animals were obtained from Charles River laboratories (Wilmington, MA) and housed in an air-conditioned room (23 ± 2°C) with ad libitum feed and water on a 12:12-h light-dark cycle. After a brief acclimatization period, adult DIO and DR females were housed overnight with their respective male counterparts. Copulation was confirmed by the presence of sperms in the vaginal smears obtained on the following morning. The day when the smears were positive for the presence of sperms was considered as day 1 of gestation. Weekly body weight increments were also used to further confirm the gestational status. Nonpregnant females were eliminated from the study. Pregnant DIO (n = 8) and DR animals (n = 9) were divided into two groups each: control (normal pregnancy) and prenatal stress (stressed during pregnancy). Animals in the control group were undisturbed throughout the gestational period. Dams in the prenatal stress group were subjected to restraint stress from day 14 to day 21 of gestation. Briefly, the animals were restrained in transparent glass cylinders for 45 min three times/day. To prevent acclimatization to the time of restraint stress, variable time restraint stress protocol was followed (4). According to this protocol, the time of restraint stress was randomly shifted every day between certain time periods: 9–11 AM (morning session), 12–2 PM (afternoon session), and 4–6 PM (evening session).

Offspring housing and dietary treatment. Birth weight of the DIO and DR offspring were recorded within 24 h of birth. To avoid variability due to postnatal nutrition, litter size was normalized to 8 pups per mother (4 males and 4 females) in all of the groups. Male offspring were weaned onto chow diet at 3 wk of age. Weekly body weight and food intake measurements were recorded. Caloric intake (means ± SE, kcal) for each week was calculated by multiplying the average food intake per week by the caloric content of the chow diet (3.11 kcal/g). At the end of 9 wk, the offspring from each mother were subdivided into two groups (yielding n = 8, 2 from each dam), housed separately, and randomly assigned to chow (25% protein, 72% carbohydrate, and 5% calories as fat with an energy density of 3.11 kcal/g) or HF diet (20% protein, 35% carbohydrate, and 45% calories as fat with an energy density of 4.73 kcal/g; Research Diets, New Brunswick, NJ). After 1 wk of chow or HF diet treatment, offspring were euthanized by decapitation. All procedures involving animals were in compliance with the National Institutes of Health’s Guide For the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Trunk blood and liver were collected at the time of euthanization. Blood glucose levels were measured using a glucometer. Serum was separated from trunk blood and stored at −80°C until further analysis. Visceral adipose tissue (VAT) was also collected, and the samples were assayed in duplicate per the manufacturer’s instructions.

Insulin and C-peptide measurements. Serum Insulin and C-peptide levels were measured using a double-antibody RIA kit (Millipore, Billerica, MA), and the samples were assayed in duplicate per the manufacturer’s instructions.

Western blotting for CEACAM1. Liver (50–100 mg) was homogenized in lysis buffer (150 mM NaCl, 50 mM HEPES, 5% sodium azide in water) containing protease inhibitors (100 mM PMSF, 5 mM Sodium Orthovanadate–Complete tablets; Roche), and equal amounts of protein (30 μg) were resolved on a 7% SDS-PAGE and immunoblotted with 1:500 custom-made anti-CEACAM1 α-P3 (Ex) antibody, raised in rabbit against three peptides (Ex: amino acids (aa) 49–64; Y488: aa 482–495; and Y513: aa 506–519), and affinity-purified against the extracellular peptide (Ex: aa 49–64). Membranes were reprobed with 1:1,000 mouse anti-GAPDH (Santa Cruz Biotechnol-ogy, Santa Cruz, CA) for normalization. Following detection by chemiluminescence, band intensity was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Adipocyte area measurement. Visceral adipose tissue was collected at the time of euthanization, stored in 10% neutral buffered formalin for 3 days, transferred to 70% ethanol prior to being embedded in paraffin blocks, and sectioned (5 μm). One representative section from each animal was stained with hematoxylin and eosin and used for adipocyte area measurements. The adipocyte area measurements were done using an NIS element microscope under ×20 magnification. The adipocyte area was measured at three random sites in each animal present in the group. The data are represented as the mean adipocyte area (μm²) per group.

Statistical analysis. Birth weight and weaning weight were analyzed by two-way ANOVA to study the interaction between genotype and prenatal stress followed by post hoc Fisher’s least significant difference (LSD) test. Changes in weekly body weight and calorie intake were analyzed using repeated-measures ANOVA followed by post hoc Fisher’s LSD test. Metabolic parameters after 1 wk of HF diet treatment were analyzed using three-way ANOVA to study the interaction between genotype, diet, and prenatal stress, followed by post hoc Fisher’s LSD test. Results were considered to be significant when P < 0.05.

RESULTS

Birth and weaning weights. Prenatal stress did not affect litter size or sex ratio in either DIO or DR rats. Birth and weaning weights (means ± SE, g) of DIO and DR offspring of all treatment groups are shown in Fig. 1, A and B, respectively. Prenatal stress decreased birth weight significantly in the DR offspring (6.2 ± 0.1 vs. 6.7 ± 0.12, P < 0.05) but not in the DIO offspring (5.5 ± 0.1 vs. 5.7 ± 0.1) when compared with nonstressed controls. Interestingly, DIO offspring (both stressed and nonstressed) had lower birth weights compared with the DR groups (P < 0.05). We observed a significant genotype and prenatal stress effect (F = 66.3 and 10.6, respectively, P < 0.05) with respect to birth weight.

Prenatally stressed DR animals, which had low birth weight, gained weight to reach control levels at weaning. Also, DIO animals gained weight more rapidly and weighed significantly more than DR animals at the time of weaning. Two-way ANOVA analysis revealed a significant genotype effect (F = 12.61, P < 0.05) with respect to weaning weight.

Fig. 1. Effects of prenatal stress on birth weight and weaning weight in the dietary resistant (DR) and diet-induced obese (DIO) male offspring. Birth weight (means ± SE, g; A) and weaning weight (means ± SE, g; B) of male DR and DIO offspring whose dams underwent normal gestation (controls) and prenatal stress (restraint stress, 3 times daily from days 14 to 21 of gestation) are shown. *P < 0.05 compared with nonstressed DR offspring (stress effect); $P < 0.05 compared with DR groups (genotype effect).
Postweaning body weight gain and calorie intake. Postweaning body weight gain (means ± SE, g) in DIO and DR animals from weaning to week 9 is shown in Fig. 2A. Although both DIO and DR animals gained weight during the postweaning period, the rate of BW gain was very different between the two groups. During the early postweaning period, BW gain in nonstressed DR offspring was (30.5 ± 0.41) at week 4, whereas BW gain in stressed DR offspring was slightly higher (32.4 ± 0.49). At week 5 and beyond, the BW gains in nonstressed and prenatally stressed DR animals were not different from each other. In contrast, BW gain in DIO offspring was significantly higher from weeks 4 to 9 compared with DR rats. Moreover, prenatally stressed DIO offspring had significantly lower BW gain at weeks 4 (34.7 ± 0.6) and 5 (82.8 ± 1.4) compared with nonstressed DIO offspring (36.9 ± 0.7 and 89.5 ± 1.5 in weeks 4 and 5, respectively, P < 0.05). However, beyond week 5, BW gain in DIO offspring was very similar between the stressed and nonstressed groups. Two-way repeated-measures ANOVA indicated a significant genotype effect (F = 15.7, P < 0.05) with respect to postweaning weight gain on a chow diet.

Calorie intake of the offspring during the post-weaning period is shown in Fig. 2B. The average calorie intake (means ± SE, kcal/day) in DIO rats was significantly higher than that in DR animals from weeks 4 to 9. Prenatal stress caused DR rats to consume more calories at weeks 4 and 5 compared with the nonstressed group. Prenatally stressed DIO animals consumed fewer calories in week 4 compared with controls (216 ± 5.3 vs. 231.3 ± 3.1, P < 0.05). After week 5, no differences in calorie intake were observed between the stressed and nonstressed groups in the DIO or DR animals. There was a significant effect of genotype (F = 35.01, P < 0.05) on calorie intake in DIO and DR animals.

Changes in body weight and calorie intake after 1 wk of HF diet. Final BW, BW gain, and average calorie intake after 1 wk of HF diet exposure in DIO and DR animals are presented in Table 1. Both DIO and DR animals had higher BW gain, final BW, and calorie intake when placed on a HF diet. Prenatal stress had a different effect on the two genotypes. Whereas in DR rats prenatal stress appeared to produce modest to no changes in these parameters, in DIO animals prenatal stress caused a modest decrease and a marked rebound effect when the stressed DIO animals were placed on a HF diet.

Visceral adipose tissue/BW ratio and adipocyte area. VAT/BW ratio and adipocyte area (means ± SE, µm²) in the DIO and DR groups are shown in Fig. 3, A and B, respectively. As expected, HF diet treatment increased VAT/BW ratio in the nonstressed and prenatally stressed DIO and DR groups. However, the increase in VAT/BW ratio in DIO animals on the HF diet was significantly higher compared with DR animals on the HF diet (Fig. 3A). We observed a significant diet (F = 142.7, P < 0.0001) and genotype (F = 30.4, P < 0.0001) but no prenatal stress effect on VAT/BW ratio.

HF diet increased adipocyte area in the DIO offspring (both controls and stressed) but not in DR animals (Fig. 3B). Also, prenatally stressed DIO animals on chow diet had larger adipocytes than nonstressed DIO rats. Placing animals on a HF diet eliminated this difference in adipocyte size between

Table 1. Effects of prenatal stress on final BW (g), BW gain (g), and average calorie intake/wk (kcal) after 1 wk of HF diet exposure in the DR and DIO offspring are tabulated.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DIO-Control</th>
<th>DIO-Stress</th>
<th>DR-Control</th>
<th>DR-Stress</th>
<th>P Value (S × D × G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final BW, g</td>
<td>387.2 ± 11.8$</td>
<td>408.9 ± 8.4$</td>
<td>372.2 ± 4.25</td>
<td>415.9 ± 7.4$</td>
<td>293.2 ± 7.1</td>
</tr>
<tr>
<td>BW gain, g</td>
<td>40.9 ± 4.3$</td>
<td>56.1 ± 2.0$</td>
<td>29.5 ± 2.8</td>
<td>57.1 ± 3.4$</td>
<td>28.0 ± 1.6</td>
</tr>
<tr>
<td>Calorie intake, kcal/wk</td>
<td>732.3 ± 57.2$</td>
<td>946.2 ± 24$</td>
<td>656.7 ± 30.6</td>
<td>998.5 ± 34$</td>
<td>475.4 ± 14.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. BW, body weight; CH, chow; HF, high-fat; DR, dietary resistant; DIO, diet-induced obese; S, stress; D, diet; G, genotype. Control offspring are from nonstressed dams, and stressed offspring are from dams that underwent chronic restraint stress during pregnancy. Significant difference (P < 0.05) between HF and their respective CH groups (diet effect); Significant difference (P < 0.05) from DR groups (genotype effect), *significant difference (P < 0.05) from DR control (stress effect). P value for the interaction between S, D, and G is also provided.

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stressed and nonstressed DIO animals. There was a significant diet ($F = 40.8, P < 0.0001$) and genotype ($F = 22.8, P < 0.0001$) effect on adipocyte area. The fact that DR animals on HF diet had higher fat mass without an increase in adipocyte size suggests that there was ongoing adipose hyperplasia rather than hypertrophy, as seen in DIO animals.

Blood glucose, serum insulin, and C-peptide levels. Blood glucose (means $\pm$ SE, mg/dl; $A$), serum insulin (means $\pm$ SE, ng/ml; $B$), and serum C-peptide levels (means $\pm$ SE, pM; $C$) in all treatment groups after 1 wk of chow or HF diet treatment are shown. Control represents offspring from nonstressed dams, and stress represents offspring from dams who underwent restraint stress during pregnancy. #Significant difference ($P < 0.05$) from their respective chow-fed counterparts (diet effect); $SP < 0.05$ from all of the other DR groups (gene effect); $*P < 0.05$ from stressed DR groups (chow and HF) and chow-fed DR control group.
blood glucose levels significantly in all the DIO and DR groups, irrespective of prenatal stress (Fig. 4A). No changes in insulin levels were observed in the DR groups. In contrast, serum insulin levels in prenatally stressed DIO offspring on the HF diet (26.2 ± 11.9, $P < 0.05$) were significantly greater compared with all other DIO and DR groups (both chow- and HF-fed groups) (Fig. 4B). There was a significant effect of prenatal stress ($F = 5.6, P < 0.05$), genotype ($F = 7.2, P < 0.01$), diet ($F = 9.7, P < 0.005$), and stress-genotype-diet interaction ($F = 5.9, P < 0.05$) with respect to serum insulin levels. Serum C-peptide levels also showed a pattern similar to insulin levels. After 1 wk of the HF diet, prenatally stressed DIO animals had significantly higher C-peptide levels compared with all of the other groups (Fig. 4C). The stress-genotype-diet interaction with respect to serum C-peptide levels was close to significance ($F = 3.96, P = 0.05$).

Liver CEACAM1 levels. Western blot analysis revealed that there were no changes in hepatic CEACAM1 protein levels in DR animals, regardless of diet or stress (Fig. 5). However, in DIO animals, CEACAM levels were lower in nonstressed compared with DR animals. CEACAM1 levels in prenatally stressed DIO rats on chow were comparable with DR rats; however, exposure to the HF diet produced a dramatic decrease in CEACAM1 expression in stressed DIO offspring ($P < 0.05$).

DISCUSSION

It is now well established that intrauterine perturbations may have long-lasting health consequences in the offspring (11, 12, 38). Several epidemiological studies have associated compromised in utero environment with increased incidence of obesity, type 2 diabetes, and hypertension in adulthood (15, 20, 21, 23, 36). These effects are likely to be more apparent when such offspring are exposed to high-energy diets as adults. With escalating rates of maternal obesity and juvenile obesity, it is important to understand whether genetic predisposition to obesity would alter the offspring’s susceptibility to dietary challenges in later life. Therefore, we investigated the effects of a combination of prenatal stress and HF diet challenge on metabolic parameters in DIO offspring that are genetically susceptible to obesity. DR offspring that are resistant to the development of obesity were used for comparison.

Several studies have demonstrated that prenatal stress is capable of producing a dramatic reduction in birth weight, suggesting intrauterine growth retardation (IUGR) (9, 25, 34, 40). This is strongly suspected to contribute to the development of obesity in later life (39). In our study, DIO offspring had significantly lower birth weights compared with DR animals, indicating that IUGR that is observed in this model could play a vital role in the development of obesity. Prenatal stress produced a significant reduction in birth weight in DR but not DIO offspring. This is understandable given the fact that DIO offspring had low birth weight to begin with, and any further reduction in body weight might have been incompatible with survival. To our knowledge, this is the first time that birth weight has been reported in DIO and DR rats, and our results indicate that IUGR in the DIO rat model could be an inherent characteristic predisposing these offspring to obesity.

Although many studies have reported prenatal stress-induced reduction in birth weight (9, 25, 34, 40), few studies have also reported no change (5, 16) or increase in birth weight (42). The difference in the stress protocol (type of stressor, intensity, and the timing of the stress procedure) could explain the disparity in findings between studies. The mechanism by which prenatal stress causes IUGR is not clear. Restraint stress during the last week of gestation reduces food intake in dams (28, 43) and produces weight loss (28, 30). Moreover, stress-induced increases in glucocorticoid levels could also play a role in this phenomenon since stressed dams have higher levels of stress hormones (41) or increased adrenal weight (28). Although we did not measure food intake or glucocorticoid levels in dams in this study, there is a strong likelihood that these could have played a role in decreasing birth weight in DIO and DR rats.

Although prenatal stress affected birth weight in DR offspring, it did not seem to affect postnatal body weight gain. There were early differences in weight gain after weaning, but these disappeared once the pups reached 5 wk of age. However, during this period, DIO pups gained weight more rapidly than DR pups. Challenging the pups for a very short period (1 wk) with HF diet exposure produced a significant gain in body weight in both stressed and nonstressed DIO animals, and this was accompanied by a corresponding increase in calorie intake as well. However, in DR offspring, although calorie intake increased, there was no corresponding increase in body weight, suggesting a possible higher rate of metabolism in this model.

Disturbances in insulin sensitivity in prenatally stressed offspring have been reported previously in both humans and rodents (13, 25, 31, 33). In accord with these findings, we observed hyperinsulinemia after 1 wk of HF intake in prena-
tally stressed DIO offspring but not in the DR offspring. Hyperinsulinemia could result from increased insulin secretion or reduced insulin clearance (22). C-peptide is a reliable marker of insulin secretion. It does not undergo first-pass effect like insulin and has a longer half-life than insulin. C-peptide levels in DR rats were comparable with that seen in normal Sprague-Dawley rats. However, in DIO rats, prenatal stress in combination with HF diet produced a marked increase in C-peptide levels, and this was substantiated by higher levels of insulin in this treatment group as well. The increase in C-peptide levels could be due to a direct effect of fatty acids present in the HF diet on β-cells of the pancreas (44). Besides increased insulin secretion in this group, there was also a marked reduction in CEACAM1 levels compared with the corresponding chow-treated group, which was suggestive of reduced insulin clearance. The increased secretion of insulin combined with its reduced clearance by the liver could be at least partially responsible for the marked increases in insulin levels observed in the HF-fed, stressed DIO offspring. This is supported by studies in mice with global or liver-specific inactivation of CEACAM1 that exhibit hyperinsulinemia and insulin resistance resulting from impairment of insulin clearance (8, 35).

To our knowledge, this is the first report demonstrating prenatal stress-induced changes in liver CEACAM1 expression in the offspring that results in hyperinsulinemia in response to a short-term challenge with HF diet. Further studies are needed to identify the mechanisms underlying prenatal stress-induced programming of CEACAM1 expression.

CEACAM1 levels were relatively higher in the DR group irrespective of prenatal stress or HF diet exposure. This agrees well with the levels of insulin seen in DR rats. However, in the DIO group, there is lack of correlation between CEACAM1 and insulin levels in nonstressed animals. It is likely that fatty acids that increase due to HF diet exposure act directly on β-cells of the pancreas to stimulate C-peptide and insulin secretion. This suggests fundamental differences in CEACAM1 expression between DIO and DR rats. The impact of these differences on metabolic functions in these models needs further investigation.

In summary, the present study suggests that prenatal stress programs the offspring for adverse metabolic outcomes. It decreases birth weight and promotes postnatal catchup growth. Furthermore, challenge with a HF diet even for a short duration of 1 wk causes hyperinsulinemia, especially in DIO rats. An increase in C-peptide levels and a reduction in CEACAM1 levels are possible mediators of this effect. This is most likely to set the stage for adulthood disorders such as type 2 diabetes and obesity in this model. HF diet challenge affects adipose tissue differentially in the DIO and DR model; whereas it induces hypertrophy in DIO rats, it causes hyperplasia in DR animals. Nonetheless, we provide evidence that preexisting genetic predisposition to obesity increases the susceptibility of the offspring to the metabolic programming effects of prenatal stress.

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