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## Diet-induced obesity impairs mammary development and lactogenesis in murine mammary gland

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**Flint, David J, Maureen T Travers, Michael C Barber, Nadine Binart, and Paul A Kelly.** Diet-induced obesity impairs mammary development and lactogenesis in murine mammary gland. *Am J Physiol Endocrinol Metab* 288: E1179–E1187, 2005. First published January 25, 2005; doi:10.1152/ajpendo.00433.2004.—We have developed a mouse model of diet-induced obesity that shows numerous abnormalities relating to mammary gland function. Animals ate ~40% more calories when offered a high-fat diet and gained weight at three times the rate of controls. They exhibited reduced conception rates, increased peripartum pup mortality, and impaired lactogenesis. The impairment of lactogenesis involved lipid accumulation in the secretory epithelial cells indicative of an absence of copious milk secretion. Expression of mRNAs for  $\beta$ -casein, whey acid protein, and  $\alpha$ -lactalbumin were all decreased immediately postpartum but recovered as lactation was established over 2–3 days. Expression of acetyl-CoA carboxylase (ACC)- $\alpha$  mRNA was also decreased at parturition as was the total enzyme activity, although there was a compensatory increase in the proportion in the active state. By *day 10* of lactation, the proportion of ACC in the active state was also decreased in obese animals, indicative of suppression of *de novo* fatty acid synthesis resulting from the supply of preformed fatty acids in the diet. Although obese animals consumed more calories in the nonpregnant and early pregnant states, they showed a marked depression in fat intake around *day 9* of pregnancy before food intake recovered in later pregnancy. Food intake increased dramatically in both lean and obese animals during lactation although total calories consumed were identical in both groups. Thus, despite access to high-energy diets, the obese animals mobilized even more adipose tissue during lactation than their lean counterparts. Obese animals also exhibited marked abnormalities in alveolar development of the mammary gland, which may partially explain the delay in differentiation evident during lactogenesis.

high fat; acetyl-coenzyme A carboxylase; morphogenesis

IN 1997, THE WORLD HEALTH ORGANIZATION officially declared human obesity to be one of the most significant health problems facing mankind. In the United States, for example, the prevalence of obesity trebled between 1974 and 1994 (17). In 1998, the worldwide total of obese individuals (body mass index  $>30$ ) was 250 million. Obesity has been identified as an independent risk factor in several life-threatening diseases, including diabetes, hypertension, and cardiovascular disease (5), and has also recently been proposed to have adverse effects on breastfeeding (27) and mammary tumorigenesis (36). Indeed, a recent meta-analysis of the available epidemiological data proposed that 25% of the risk of developing breast cancer could be attributed to obesity and inactivity (40). The decision not to breastfeed has additional knock-on effects, since breast-

feeding, as opposed to bottle feeding, has been proposed to reduce the incidence of childhood obesity, and breast feeding also protects the mother from developing obesity herself (13, 16) at a time when she is at an increased risk of developing obesity (32). The effects of obesity on lactogenesis have emanated from early studies in the rat where obesity has been shown to decrease the chances of a successful outcome for pregnancy and lactation (12, 29–31). In fact, even earlier studies in heifers revealed that overfeeding led to decreased epithelial and increased adipose tissue volumes (6, 35). An effect of obesity on mammary gland function is not entirely surprising when one considers the intimate relationship that exists between adipose and mammary tissues. The mammary gland develops in a bed of white adipose tissue, and there are extensive mesenchymal-epithelial interactions in which the adipose mesenchyme “instructs” aspects of mammary epithelial development (9). However, it is worth noting that, although branching morphogenesis requires the presence of adipose tissue, development of side-branching and alveoli can occur even in its absence (8).

The relationship between mammary and adipose tissues extends, in a different context, into lactation, since adipose tissue triglycerides are mobilized to provide fatty acids for milk lipid synthesis (11). However, excessive use of long-chain fatty acids from adipose tissue in obese rats leads to an increase in both total and saturated fatty acid content of milk (29) and increased fat deposition in the offspring consuming the milk. Although such studies have examined in detail the metabolic consequences of obesity during lactation, they did not examine mammary gland development.

To begin to address the relative importance of metabolic vs. developmental effects, we have developed a diet-induced mouse model based on a simplified “cafeteria-style” high-fat diet. Studies have already been conducted in genetically obese mice, such as the leptin-deficient (*ob/ob*) mouse. These mice are obese and infertile. Treatment with leptin can render these animals fertile, and once mated, even if leptin treatment is withdrawn, successful pregnancies ensue (7, 25). However, even if leptin treatment is maintained, mammary function is impaired to the point that these animals fail to lactate. These studies imply that obesity per se rather than leptin deficiency may be responsible for problems of mammary development. Indeed, diet-induced obesity (the most prevalent form) is actually associated with increased (rather than decreased) levels of leptin, and thus we considered that the findings reported here would be more widely applicable since they more closely

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resemble the human obese condition, other than for a small number of individuals who are leptin deficient. We considered it important to undertake these studies in the mouse because a number of transgenic and knockout mouse models exist in which relevant potential causative factors (leptin, IGF-I, aromatases, plasminogen, plasminogen activators/inhibitors) have been manipulated and which could be used in subsequent investigations. Therefore, our first aim was to develop a model of diet-induced obesity to examine mammary function during pregnancy and lactation to confirm the metabolic defects exhibited in rats and to extend these findings by examining whether defects in mammary development were evident and whether, as a consequence, such a model might prove useful for studies of tumorigenesis in the longer term.

## MATERIALS AND METHODS

**Animals.** Female mice (OF1, outbred Swiss mice from Charles River) aged 4 mo were housed in an environment of 12:12-h light-dark at an ambient temperature of 22°C. They were fed either a standard laboratory diet (UAR, Epinay-sur-Orge, France) or a combination of ad libitum access to the standard diet and, in addition, ad libitum access to a high-fat diet. The standard diet, AO3, is designed for breeding animals, and its composition is shown in Table 1.

The high-fat diet consisted of three components [a high-fat diet prepared commercially by UAR, based on the standard diet AO3, but supplemented with beef tallow along with chocolate and peanuts and with equivalent quantities of vitamins and trace elements per unit weight as the standard diet (to avoid dilution by the fat component)]. The high-fat diet had the consistency of a stiff paste that was used to prepare a single mix containing coarsely fragmented chocolate and peanuts. This coarse mixture allowed animals to identify and consume the individual components separately. The ratio for the mix was based on preliminary experiments in which all of the dietary components were provided separately. Food intakes and body weights were initially monitored on a weekly basis. After 2 mo on the diet, all females were housed for 7 days with a male, and any females that did not mate successfully were reintroduced to a male 2 wk later, again for a period of 7 days. Nonpregnant animals were excluded from further study, whereas pregnant animals were housed individually, and body weight and food intakes were determined daily throughout pregnancy and lactation. Litter numbers and weight were recorded, and litter size was adjusted to eight in number at parturition. Five lean and four obese animals were killed on *day 14* of pregnancy, five lean and seven obese

animals were killed on *day 1* of lactation, and 10 lean and 8 obese animals were killed on *day 10* of lactation.

Mammary tissue was weighed and stored at -80°C. Small milk samples were obtained by exudation of the tissue and analyzed for protein and fat content. Fat content was determined by extraction of the sample two times with 4 vol of chloroform, which was then evaporated to dryness in a weighed vial. A whole mount of the fourth right abdominal mammary gland was prepared as previously described except that the glands were gently squeezed between two microscope slides because of the thickness of the glands in obese animals (37). Whole-mount images from animals killed on *day 14* of pregnancy were analyzed using Leica QWin version 2.6. The monochrome images were subjected to thresholding so that white levels >156 (representing adipose tissue) were selected. The area occupied by adipose tissue was subtracted from the total image area to provide the proportion of parenchymal tissue. The fifth left gland was frozen in optimum cutting temperature compound. The weight of adipose tissue from the parametrial site (the major site in adult female mice) was also recorded.

DNA content of the mammary gland was determined by the method of Labarca and Paigen (18) with specific methods for the mammary gland, as previously described (2).

Steady-state levels of mRNAs were determined by RNase protection assay followed by quantitation of protected fragments by phosphorImage analysis, as described previously (39). Briefly, tissue samples were powdered in a mortar and pestle using liquid nitrogen and homogenized in 5 M guanidinium isothiocyanate and 100 mM EDTA, pH 7.0, using a constant tissue-to-volume ratio. Aliquots (1–40  $\mu$ l) of these were hybridized to the antisense cRNA and subsequently digested using RNase A/T1 and proteinase K. After extraction with phenol and chloroform, the samples were precipitated two times and rinsed with 80% (vol/vol) alcohol. Samples were dried, resuspended, and, after denaturing in formamide-loading buffer at 85°C, resolved on 6% (wt/vol) acrylamide-7 M urea gels. The intensity of individual bands was determined using ImageQuant software (Molecular Dynamics). Signals were normalized to the amount of DNA in the tissue homogenate. Acetyl-CoA carboxylase (ACC)- $\alpha$  mRNA was measured using riboprobes derived from two distinct plasmid constructs that measure the relative contribution of PIA and PII transcripts to total ACC- $\alpha$  mRNA, and the relative abundance of transcripts either excluding or including a 24-nt exon encoding eight amino acids proximal to the Ser-1200 phosphorylation site of mouse ACC- $\alpha$  (1, 3). Riboprobe vectors for mouse  $\beta$ -casein have been described previously (1). cDNA for mouse  $\alpha$ -lactalbumin and whey acid protein (WAP) were prepared by PCR of lactating mammary

Table 1. *Composition of diets*

|                             | Diets    |          |           |      |   |
|-----------------------------|----------|----------|-----------|------|---|
|                             | Standard | High Fat | Chocolate | Nuts | High-Fat Mix 6:2:3<br>(high fat-chocolate-nuts) |
| Protein, g/kg               | 214      | 167      | 76        | 285  | 183   |
| Carbohydrate, g/kg          | 517      | 130      | 564       | 78   | 195   |
| Fat, g/kg                   | 52       | 600      | 297       | 530  | 526   |
| Total energy, kJ/g          | 15.5     | 29.3     | 21.9      | 25.8 | 27.0  |
| Minerals and vitamins, g/kg | 57       | 57       |           |      |   |
| Calcium, mg/kg              | 9,000    | 9,000    |           |      |   |
| Phosphate, mg/kg            | 5,900    | 5,900    |           |      |   |
| Sodium, mg/kg               | 2,800    | 2,800    |           |      |   |
| Potassium, mg/kg            | 8,600    | 8,600    |           |      |   |
| Manganese, mg/kg            | 90       | 90       |           |      |   |
| Copper, mg/kg               | 22       | 22       |           |      |   |
| Vitamin A, $\mu$ g/kg       | 14,000   | 14,000   |           |      |   |
| Vitamin D3, $\mu$ g/kg      | 1,200    | 1,200    |           |      |   |
| Vitamin E, mg/kg            | 50       | 50       |           |      |   |

Control animals received the standard diet. Animals fed the high-fat diet received the standard diet plus the high-fat mix.

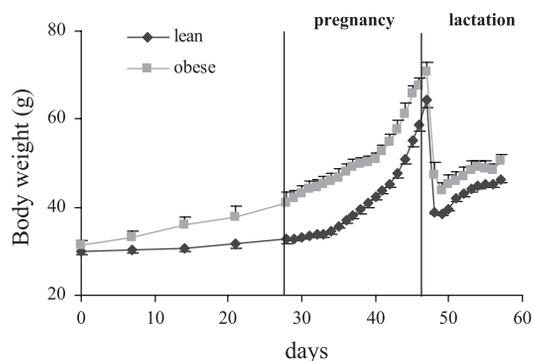


Fig. 1. Body weight changes during the reproductive cycle in mice fed a high-fat diet.

gland cDNA with primers 5'-GCCTGCCTTTCAAGCCACAGAGCT-3' and 5'-GGGGGCTCAGGGCTTCTCACAAC-3' and 5'-GGTGGC-CCTCGCTCAGAACCTAGA-3' and 5'-CAGGGCCGGCTGCT-CACTGAA-3', respectively, and cloned into the pGEM-7zf(+) vector (Promega) for riboprobe synthesis.

Total ACC activity and the proportion in the active state were determined as previously described.

**Statistical analyses.** Comparisons were made either using Student's unpaired *t*-test or, where multiple comparisons were made, by ANOVA followed by post hoc tests involving the Bonferroni correction. Mortality and conception rates were compared using Fischer's exact test.

## RESULTS

**Body weight and food consumption.** Feeding a high-fat diet to mice led to an increase in body weight ( $P < 0.01$ ) of  $12.0 \pm 1.2$  (SE) g in the high-fat group compared with  $4.7 \pm 0.3$  g in controls during the 5 wk before mating. Calorie intake increased from 65 to 100 kJ/day in the high-fat group during this period, whereas it remained constant in the controls (Figs. 1 and 2). During pregnancy, the controls increased their food intake progressively from  $75 \pm 4$  (SE) kJ/day on day 1 to  $118 \pm 9$  kJ/day on day 18 of pregnancy, whereas the high-fat diet group initially increased their intake from  $106 \pm 2$  to  $173 \pm 39$  kJ/day on day 8 of pregnancy but then showed a precipitous decline to  $104 \pm 24$  kJ/day on day 10 of pregnancy (exclusively due to a decrease in intake of the high fat diet) before intakes increased again to achieve values of  $153 \pm 14$  kJ/day on day 18 of pregnancy (Fig. 2).

Total body weight increased from  $33 \pm 1$  to  $64 \pm 5$  g in controls and from  $42 \pm 2$  to  $71 \pm 2$  g in the high-fat diet group

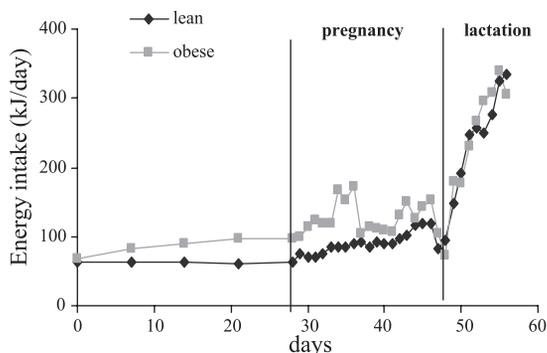


Fig. 2. Energy intake during the reproductive cycle in mice fed a high-fat diet.

during pregnancy. Between day 18 of pregnancy and day 2 of lactation, the controls had food intakes of  $118 \pm 9$  (P18),  $83 \pm 7$  (parturition, L1), and  $96 \pm 13$  kJ/day (L2), whereas the high-fat diet group exhibited a much greater decline in food intake, with intakes, respectively, of  $154 \pm 14$ ,  $104 \pm 20$ , and  $73 \pm 11$  kJ/day. After the initial large decrease in body weight after delivery of the young, the decline in food intake was accompanied by a further decrease in body weight during the first day postpartum of  $3.7 \pm 1.2$  g (mean  $\pm$  SE) in the high-fat animals, which was significantly greater than that of the controls ( $0.2 \pm 0.6$  g,  $P < 0.01$ ; Fig. 1). Food intake increased dramatically in the control animals from  $96 \pm 13$  kJ/day on day 1 to  $334 \pm 13$  kJ/day on day 10 of lactation. High-fat diet animals also increased their intake from  $74 \pm 11$  to  $336 \pm 34$  kJ/day. There were also switches in the proportion of energy derived from protein, carbohydrate, and fat. Whereas in control animals the percentages of calories derived from protein, carbohydrate, and fat were (respectively) 25:62:13, in obese animals it was 12:17:71 during early pregnancy, 16:30:54 in late pregnancy, 14:20:66 in early lactation, and 15:25:60 in late lactation. Thus, during late pregnancy, there was an increase in the proportion of energy derived from both protein and carbohydrate (46% of calories in late pregnancy compared with 29% in early pregnancy). However, this effect was reversed within 24 h of parturition because of an increase in fat consumption. Fat intake thereafter provided 60–66% of calories throughout lactation, despite ad libitum access to the control, high-carbohydrate diet.

**Pregnancy and pup growth.** Twenty-two of 25 control animals became pregnant. In contrast, only 19 of 35 high-fat diet animals became pregnant, a difference that was statistically different ( $P = 0.0102$ , Fischer's exact test). Of 184 pups born to control animals, there was only one born dead, whereas 17/161 pups were born dead to high-fat animals, which was also a significant increase in mortality rate ( $P < 0.001$ , Fischer's exact test). At parturition, litter size was  $12.6 \pm 0.7$  (SE) g in lean and  $9.6 \pm 1.2$  g in obese animals ( $P < 0.01$  Student's *t*-test) animals, and the mean pup weight was  $1.65 \pm 0.03$  (SE) g vs.  $1.55 \pm 0.04$  g, respectively ( $P < 0.05$ , Student's *t*-test).

**Adipose tissue.** It could be inferred that the increase in body weight in high-fat-diet animals was largely the result of increased body fat, since the parametrial site alone was fivefold greater in the high-fat group on day 14 of pregnancy (Fig. 3). The amount of adipose tissue at this site decreased slightly by day 1 of lactation but decreased dramatically (by  $\sim 85\%$ )

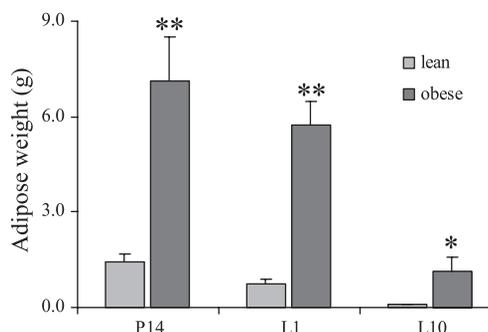


Fig. 3. Changes in parametrial adipose tissue weight during pregnancy (P14) and lactation (L1, L10) in mice fed a high-fat diet. Values are means  $\pm$  SE. \* $P < 0.05$  and \*\* $P < 0.01$ , obese vs. lean.

during lactation, although the loss of parametrial adipose tissue was quantitatively much greater in the high-fat group (4.7 g) compared with the controls (0.6 g).

Nevertheless, the amount of adipose tissue remained significantly higher in the high-fat group [ $1.12 \pm 0.48$  (SE) g] compared with the controls ( $0.10 \pm 0.01$  g,  $P < 0.01$ ).

**Mammary gland DNA content.** The weight of the mammary gland was significantly greater in the high-fat group on *day 14* of pregnancy, but the DNA concentration was approximately one-half of that of the controls. As a consequence, the total DNA content was not different (Fig. 4).

**Mammary gland function.** The high-fat-diet animals exhibited an impaired lactogenesis, as indicated by the poor initial pup growth rate of  $0.5 \pm 0.3$  (SE) g/day compared with  $2.1 \pm 0.3$  g/day in the controls ( $P < 0.05$ ; Fig. 5). This impairment was attenuated over the next 2–3 days, and subsequently pup growth rate was similar in both groups.

The delay in lactogenesis was accompanied by a number of changes in mRNA expression that were consistent with a decrease in milk synthesis. Expression of  $\alpha$ -lactalbumin,  $\beta$ -casein, and WAP were all reduced on *day 1* of lactation, whether expressed per unit of DNA or per gland, although  $\beta$ -casein and WAP were normalized by *day 10* of lactation, whereas expression of  $\alpha$ -lactalbumin remained significantly depressed (Fig. 6). These changes in mRNA expression of major milk proteins were accompanied by a significant decrease in the total protein content of the milk of obese animals ( $5.0 \pm 0.4$ ) compared with lean animals [ $6.7 \pm 0.6$  (SE),  $P < 0.05$ ].

ACC- $\alpha$  mRNA expression is driven from two promoters (PIA, principally considered to be used in liver and adipose

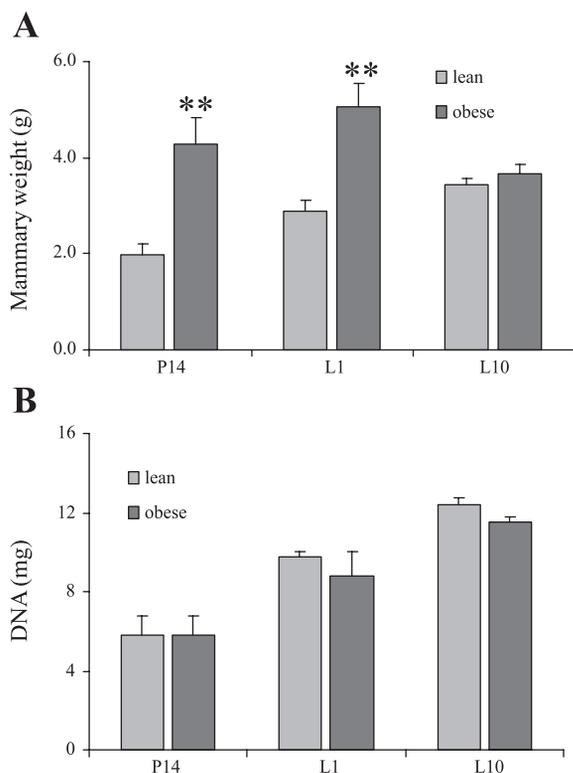


Fig. 4. Mammary gland weight (A) and DNA content (B) during pregnancy and lactation in mice fed a high-fat diet. Values are means  $\pm$  SE. \*\* $P < 0.01$ , obese vs. lean.

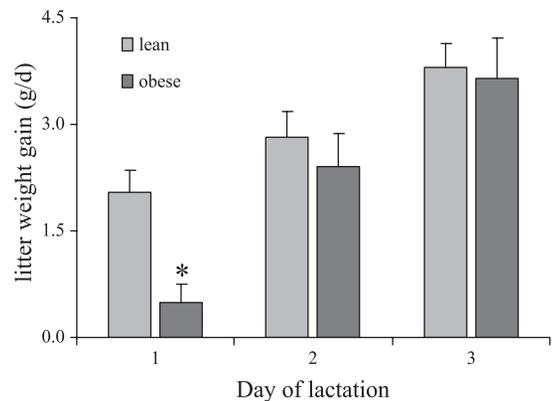


Fig. 5. Litter weight gain immediately postpartum in mice fed a high-fat diet. Values are means  $\pm$  SE. \* $P < 0.05$ , obese vs. lean.

tissue, and PII, which is more ubiquitously expressed and is considered to be the principal promoter in the mammary gland). Although PII was the major transcript in our studies, there was a fivefold increase in the expression of both PIA (reannotated from PI to conform with the human annotation; see Ref. 38) and PII transcripts between *day 14* of pregnancy and *day 10* of lactation (Table 1). On *day 1* of lactation, steady-state mRNA levels of ACC transcripts from the PII promoter were lower in obese animals, although they were normalized by *day 10* of lactation.

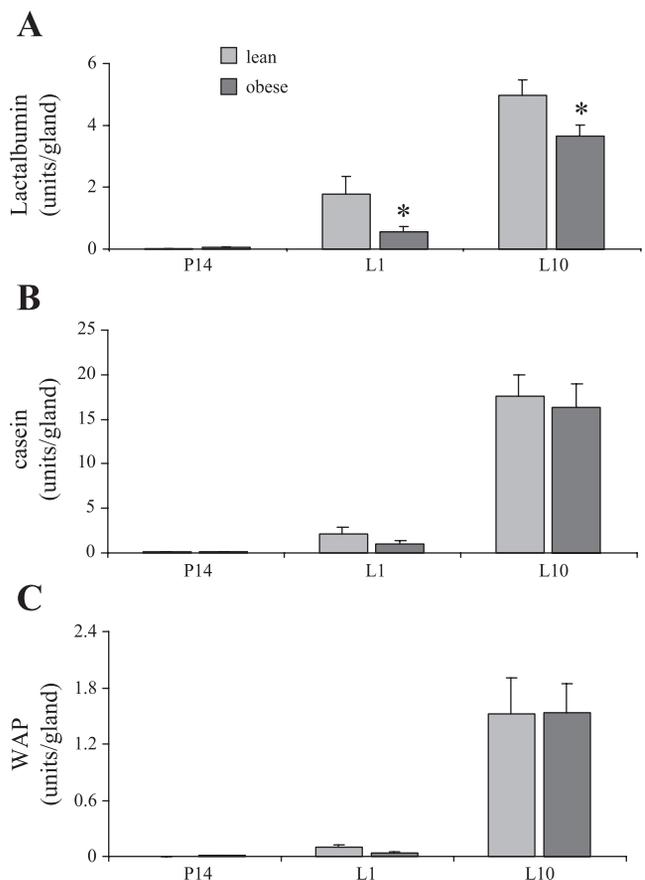


Fig. 6.  $\alpha$ -Lactalbumin (A),  $\beta$ -casein (B), and whey acid protein (WAP, C) expression during pregnancy and lactation. Values are means  $\pm$  SE. \* $P < 0.05$ , obese vs. lean.

Hybridization of a cRNA corresponding to the nucleotide sequence encompassing the region of ACC- $\alpha$  mRNA, encoding the Ser-1200 phosphorylation motif (1, 3) to lactating mammary gland RNA, produced protected fragments of 438 and 382 nucleotides (results not shown). These correspond to ACC- $\alpha$  mRNAs either containing (438 nt species, long transcript) or lacking (382 nt, short transcript) a 24-nt exonic sequence. A switch in the short/long form mRNA ratios is characteristic of the onset of lactogenesis (3). The ratio of short to long form of ACC- $\alpha$  mRNA increased from  $2.4 \pm 0.8$  (mean  $\pm$  SE) on *day 1* of lactation to  $8.0 \pm 0.1$  on *day 10* of lactation in lean mice. It also increased in obese animals, although the ratios were smaller ( $1.5 \pm 0.4$  and  $5.0 \pm 0.9$ , respectively). As a consequence, the expression of the major short transcript (ACC- $\alpha$ ) was significantly decreased in obese animals ( $52 \pm 16$  units) compared with controls ( $98 \pm 18$ ) on *day 10* of lactation ( $P < 0.05$ ; Table 2).

The total amount of ACC enzyme increased >30-fold between pregnancy *day 14* and *day 10* of lactation in control mice. ACC activity was decreased in obese animals, when compared with controls, on both *days 1* and *10* of lactation, although the difference was only statistically significant on *day 10* of lactation (Fig. 7).

The decrease in total ACC was offset on *day 1* of lactation, but not *day 10*, by an increase in the percentage of enzyme in the active site [ $34 \pm 9$  vs.  $71 \pm 7\%$  (mean  $\pm$  SE,  $P < 0.01$ ) in lean and obese animals, respectively]. Thus the total amount of enzyme in the active state on *day 1* of lactation was equivalent to control animals. These decreases in expression of ACC activity, indicative of suppression of de novo synthesis of fatty acids, were nevertheless accompanied by an increase in the lipid content of the milk of obese animals ( $21.8 \pm 4.7\%$ ) compared with lean animals ( $8.6 \pm 0.8\%$ , mean  $\pm$  SE,  $P < 0.01$ ).

**Mammary gland development.** Given the abnormal features of lactogenesis, we examined various parameters of mammary development and function in high-fat-diet animals. We noted several abnormal aspects to the glands of such animals. Whole-mount analysis showed abnormal side-branching and alveolar development by *day 14* pregnancy (Fig. 8). Furthermore, quantitative analysis of mammary development, based on whole-mount images, revealed that there was significantly more parenchyma per unit area ( $54.4 \pm 3.3\%$ , mean  $\pm$  SE) in lean animals than in obese animals ( $37.2 \pm 3.8$ ,  $P < 0.01$ ). However, because the volume of the glands in obese animals

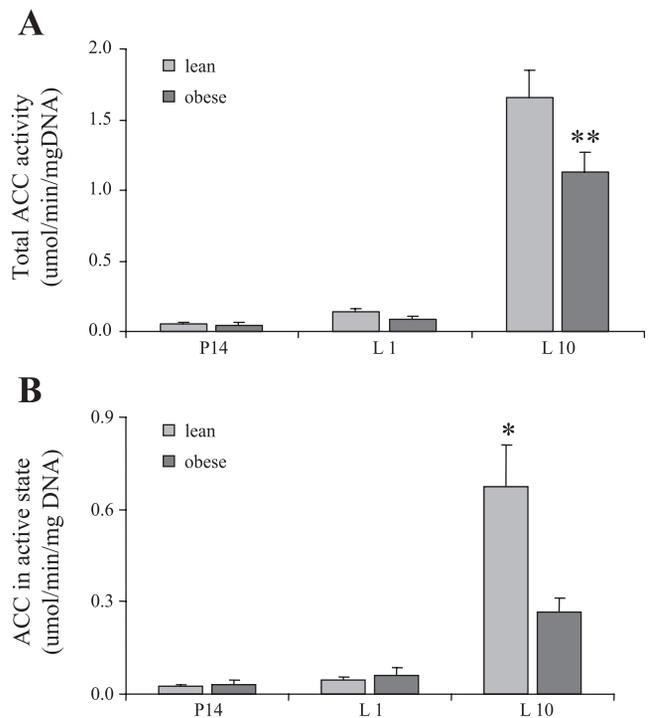


Fig. 7. Total acetyl-CoA carboxylase (ACC) activity (A) and the proportion of ACC in the active state (B) during pregnancy and lactation. Values are means  $\pm$  SE. \* $P < 0.05$  and \*\* $P < 0.01$ , obese vs. lean.

was greater, the total amount of parenchyma was not significantly different [ $1.37 \pm 0.21$  (SE) g in lean vs.  $1.51 \pm 0.25$  g in obese animals]. These findings support the observation that the total DNA content of the mammary gland was not different in lean and obese animals and that the differences in the proportion of parenchyma were most likely because of increased amounts of adipose tissue in obese animals. This in turn adds further support to the proposal that the impairment of lactogenesis was because of impaired alveolar development and/or differentiation and not because of a decrease in proliferation and, as a consequence, a reduced parenchymal mass.

Even the most severely obese animals, which demonstrated the most abnormal phenotype (greatly reduced branching frequency), exhibited ductal structures that invaded all of the mammary fat pad. It would thus appear that the principal morphological defect was reduced branching frequency and

Table 2. Steady-state mRNA expression for acetyl-coA carboxylase in mammary tissue during pregnancy and lactation in lean and obese mice

| Acetyl-CoA Carboxylase | Pregnancy ( <i>day 14</i> ) |                | Lactation ( <i>day 1</i> ) |                | Lactation ( <i>day 10</i> ) |                 |
|------------------------|-----------------------------|----------------|----------------------------|----------------|-----------------------------|-----------------|
|                        | Lean                        | Obese          | Lean                       | Obese          | Lean                        | Obese           |
| Short form/mg DNA      | $0.7 \pm 0.2$               | $3.1 \pm 1.0$  | $1.6 \pm 0.8$              | $1.4 \pm 0.5$  | $7.9 \pm 1.5$               | $4.5 \pm 1.4$   |
| Short form/gland       | $4.1 \pm 0.8$               | $14.7 \pm 3.6$ | $16.2 \pm 8.2$             | $12.0 \pm 3.4$ | $98 \pm 18$                 | $52 \pm 16^*$   |
| Long form/mg DNA       | $0.5 \pm 0.1$               | $1.2 \pm 0.2$  | $0.6 \pm 0.1$              | $0.9 \pm 0.1$  | $1.0 \pm 0.1$               | $0.8 \pm 0.1$   |
| Long form/gland        | $3.2 \pm 0.5$               | $5.8 \pm 0.4$  | $5.8 \pm 0.8$              | $7.7 \pm 0.8$  | $11.9 \pm 1.7$              | $9.2 \pm 1.7$   |
| Ratio (short-long)     | $2.5 \pm 0.5$               | $1.3 \pm 0.1$  | $2.4 \pm 0.8$              | $1.5 \pm 0.4$  | $8.0 \pm 0.1$               | $5.0 \pm 0.9^*$ |
| PIA/mg DNA             | $2.9 \pm 0.7$               | $4.1 \pm 0.8$  | $4.3 \pm 0.8$              | $2.9 \pm 0.2$  | $12.0 \pm 1.0$              | $12.5 \pm 1.0$  |
| PIA/gland              | $17 \pm 3$                  | $21 \pm 1$     | $43 \pm 8$                 | $27 \pm 5$     | $161 \pm 11$                | $145 \pm 12$    |
| PII/mg DNA             | $28 \pm 8$                  | $28 \pm 5$     | $53 \pm 14$                | $22 \pm 4^*$   | $159 \pm 12$                | $154 \pm 12$    |
| PII/gland              | $170 \pm 29$                | $148 \pm 24$   | $530 \pm 144$              | $211 \pm 62^*$ | $1,980 \pm 140$             | $1,773 \pm 130$ |

Values are means  $\pm$  SE. \* $P < 0.05$ , obese vs. lean.

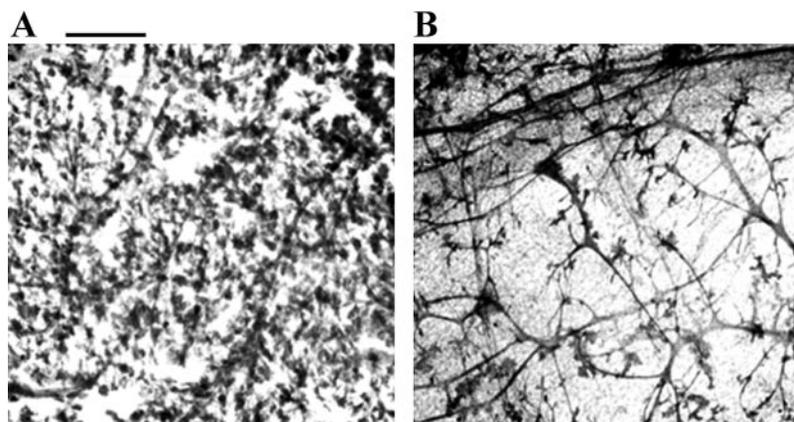


Fig. 8. Whole-mount analysis of mammary glands on *day 14* of pregnancy in lean (A) and obese (B) mice. Scale bar represents 1 mm.

impaired alveolar development, rather than a growth/proliferation defect. Although histological analyses revealed a reduction in the proportion of ductal epithelium and an increased adipocyte size in obese animals, on *day 14* of pregnancy, by *day 10* of lactation secretory alveolar structures appeared normal, although pockets of enlarged adipocytes remained in obese animals (Fig. 9). In addition, at parturition, where we had observed reduced milk secretion, the alveolar structures of obese animals exhibited dramatic accumulation of lipid within the epithelial cells (Fig. 10). Accumulation of lipid within the alveolar cells is more reminiscent of late pregnancy, where lipid synthesis precedes the copious synthesis of lactose that is responsible for the osmotic drive for water into milk.

#### DISCUSSION

In our study, we have used a simplified high-fat cafeteria-style diet to induce obesity in mice. The mice rapidly adapted to the novel diet and increased their total energy intake by ~40%. The mice were mated after 2 mo on the diet, by which time they had become obese. They exhibited numerous abnor-

malities relating to reproductive performance in terms of rate of pregnancy and stillbirths at parturition. Litter size and mean pup weight at birth were also decreased. These findings clearly indicate some impairment of reproductive performance in the obese animals, although it was not the purpose of this study to examine reproductive phenomena in detail, so no conclusions can be drawn as to the precise nature of the problem. Similar findings have been reported previously in rats (30). The first indication that mammary gland development or function might be impaired came from the observation that pup weight gain was markedly decreased during the first day of lactation. This impairment in milk production alleviated over the subsequent 2–3 days, and milk production, as evidenced by pup weight gain, was normal. This situation is reminiscent of the impaired lactogenesis that has been described in obese women (10, 27). In women, there is also an association between obesity and early cessation of breastfeeding (12, 33).

Part of the problem relating to the initiation of milk secretion may have been related to appetite, since the high-fat-diet animals lost a considerable amount of weight and ate markedly

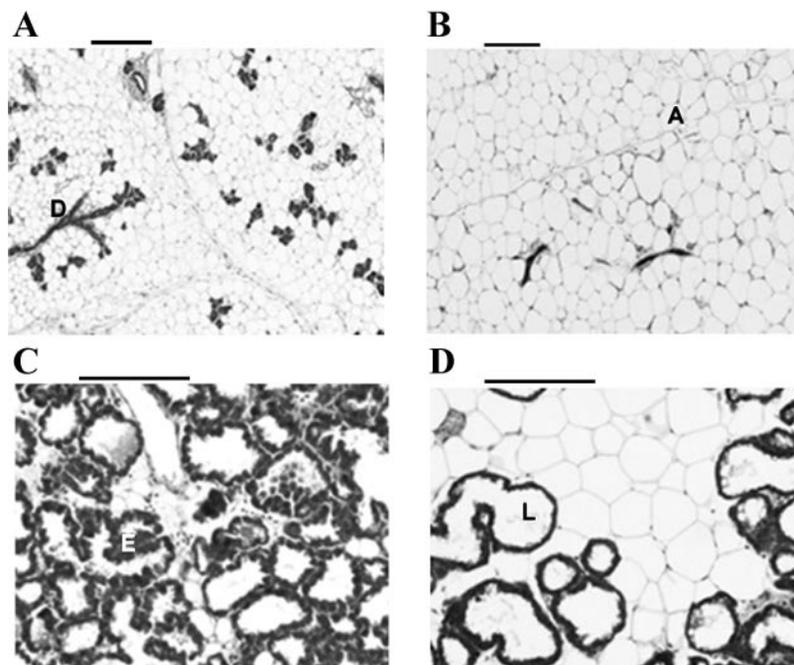


Fig. 9. Histological sections of mammary glands from pregnancy *day 14* lean (A) and obese (B) mice and from lactation *day 10* lean (C) and obese (D) mice. A, adipocyte; D, duct; E, epithelium; L, lumen containing milk. Scale bars represent 0.5 mm. (Note that magnification in C and D is two times that of A and B.)

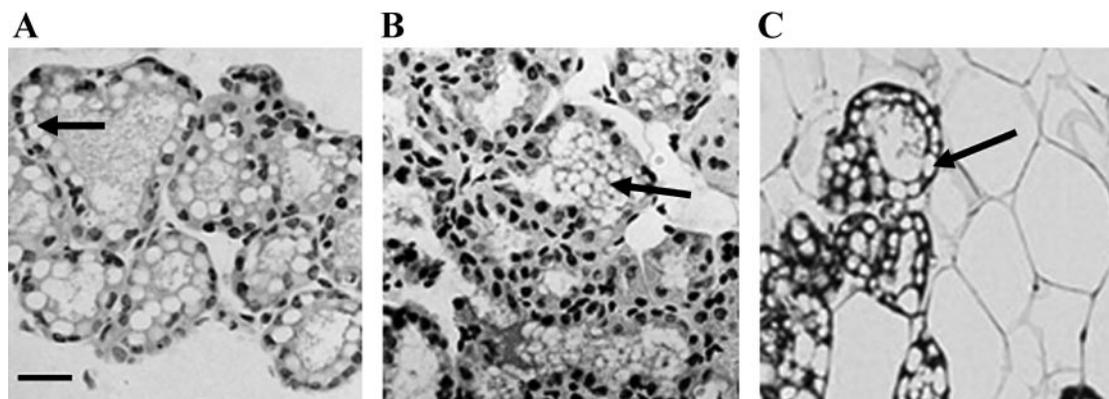


Fig. 10. Postpartum accumulation of triglycerides in alveolar epithelium of a control mouse on *day 18* of pregnancy (A) and an obese mouse on *day 1* of lactation (C) showing single lipid droplets (arrow) within numerous cells of the alveoli. By contrast, in the control mouse on *day 1* of lactation (B), lipid secretion has occurred, as evidenced by the large number of lipid droplets present in the alveolar lumen (arrow). Scale bar represents 0.1 mm.

fewer calories on the first day of lactation, at the time when they were unable to support weight gain in their pups to the same extent as in lean animals. This phenomenon of reduced food intake and greater weight loss was also evident in obese rats (31). Conflicting observations have been made regarding the possibility of improving milk output by switching to a low-fat diet during lactation, with studies showing either a further decline in milk production (29, 30) or an improvement (28). In women, one study has shown that the loss of ~0.5–1 kg body wt/wk in obese individuals did not affect the growth of their children (20–22). The reasons for these conflicting results is not immediately apparent, although it may be related to the fact that the demands of lactation in women are ~25% of total expenditure, whereas in rodents this demand can be in excess of 60%.

The provision of a high-fat diet and a source of preformed fatty acids led to reduced expression of mRNA for ACC- $\alpha$ . In particular, expression of the PII promoter was most affected at parturition, and the increased ratio of short to long forms of ACC- $\alpha$  mRNA, characteristic of mammary differentiation (3), was also impaired in obese animals. Although PII is the major promoter expressed in the mammary gland, we showed that qualitatively similar changes occurred in the expression of PIA transcripts. Earlier studies in rodents had indicated these transcripts were restricted to adipose tissue, under ad libitum feeding, and to liver, under conditions of enhanced de novo fatty acid synthesis, for instance, after high-carbohydrate feeding (15, 19). Increased expression of PIA transcripts in mouse mammary gland during lactation is unlikely to arise from the adipocyte component of the gland, since the increase in these transcripts is coincident with the increase in the epithelial cell component of mammary tissue. In support of this notion, PIA transcripts are expressed at a high level in bovine mammary epithelium during lactation (24). It is therefore possible that expression of PIA transcripts represents an important component of the enhanced fatty acid synthesis that occurs in the mammary gland during lactation, although at present the precise function of this transcript is unknown. Steady-state levels of ACC- $\alpha$  mRNA were decreased at parturition, and, consistent with this decrease, total ACC enzyme activity was also significantly decreased on *day 1* of lactation. However, somewhat surprisingly, the amount of enzyme in the active state was not affected, due to an increase in the activation state of the

enzyme. In contrast, both the total amount of ACC and the amount in the active state were significantly decreased by *day 10* of lactation. Such a decrease in ACC activity in established lactation is entirely consistent with an animal deriving large quantities of preformed fatty acids in the diet, which results in a suppression of de novo fatty acid synthesis. Although they did not measure ACC activity, this phenomenon could also be deduced from the observation of a decrease in the proportion of short-medium chain fatty acids (synthesized in the gland) compared with long-chain fatty acids (which are mainly derived from uptake of triglycerides from adipose tissue or the diet) seen in obese rats during lactation (29).

The delay in lactogenesis was accompanied by lipid accumulation within the secretory epithelial cells, and this phenomenon was very similar to that described in mice with constitutive activation of Akt, where lipid droplet size increased and lactogenesis was impaired (23, 34). The accumulation of lipid within the epithelial cells suggests a delay in the copious secretion of milk, which may relate to problems of lactose synthesis, since this is the main osmotic drive for milk secretion. The synthesis of lactose is, in turn, dependent on the synthesis of  $\alpha$ -lactalbumin, which, together with galactosyl-transferase forms the lactose synthase complex (26). We were able to show that  $\alpha$ -lactalbumin mRNA was indeed significantly impaired in obese animals at parturition and remained suppressed during lactation. Although there were small decreases in the expression of both casein and WAP on *day 1* of lactation, these were much less evident, suggesting that synthesis of the major milk proteins was not a likely cause for the impaired pup weight gain at parturition. The decrease in  $\alpha$ -lactalbumin expression in later lactation (when casein and WAP expression were normalized) suggests a decrease in milk volume. This decrease in milk volume was probably offset, however, by the increased energy content described in the milk of obese animals (29), a finding supported by our own observation of increased milk fat concentration in this study.

Although the mammary glands of animals on a high-fat diet were heavier during pregnancy and early lactation, the increase appeared to be entirely because of increased amounts of triglyceride stored in adipose tissue, since the total DNA content of the glands of obese and lean animals was equivalent at all stages examined. In fact, by *day 10* of lactation, the mammary glands of high-fat-diet animals were no longer heavier, due to

mobilization of the triglyceride from adipose tissue within the mammary gland, consistent with observations described in obese rats (30). It is important to point out that control animals also mobilized their body fat during lactation but that it was quantitatively much less important. This mobilization of body fat serves to illustrate that, at least in rodents, where there is a threefold increase in food intake during lactation and where fat mobilization provides a very small contribution to total milk energy output, mobilization is, nevertheless, preserved. It is, in fact, exaggerated in obese animals despite the fact that they have access to an extremely energy-dense diet. These animals could presumably consume more calories than the controls, but they actually did not. The reason for the defense of this mobilization strategy is not understood and may reflect an evolutionarily conserved pathway for animals that, in the wild, would more likely be underfed. In addition, there are metabolic defects, such as fatty liver syndrome (14), that lead to decreased food intakes and ketosis. Despite the fact that there were clear biochemical defects induced by a high-fat diet, we were able to demonstrate for the first time that there were also marked differences in the morphology of the mammary glands of obese mice. First, the mammary tree extended fully into the expanded fat pad, indicating increases in ductal length, which presumably occurred throughout the period on the diet. In addition, obese animals exhibited ducts with decreased branching frequency and impaired alveolar development. The degree of abnormality was most evident in the most obese animals. Clearly, such abnormal development of the mammary gland could in part be responsible for the inability to rapidly establish milk secretion.

In conclusion, this study provides a model of diet-induced obesity that exhibits many of the characteristics of mammary function in the obese woman, including impaired lactogenesis. We have confirmed and extended studies in rats by identifying impairments in the expression of  $\alpha$ -lactalbumin (lactose),  $\beta$ -casein, WAP (protein), and ACC (lipid synthesis). We also show for the first time that the PIA promoter for ACC- $\alpha$  is regulated in similar fashion to that of PII in the mammary gland of lactating mice and that enzyme activity, both total and percentage in the active state, is affected by the obese condition. Finally, we show for the first time that obesity produces abnormal mammary morphology that may contribute to the effects of the metabolic changes described. Understanding the mechanisms by which obesity influences mammary development and function are clearly important future goals.

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