Sexual Differentiation, Pregnancy, Calorie Restriction, and Aging Affect the Adipocyte-Specific Secretory Protein Adiponectin

Terry P. Combs,[1] Anders H. Berg,[1] Michael W. Rajala,[1] Simon Klebanov,[2] Puneeth Iyengar,[1] José C. Jimenez-Chillaron,[3] Mary Elizabeth Patti,[3] Sabra L. Klein,[4] Robert S. Weinstein,[5] and Philipp E. Scherer[1,6]

Adiponectin or adipocyte complement–related protein of 30 kDa (Acrp30) is a circulating protein produced exclusively in adipocytes. Circulating Acrp30 levels have been associated with insulin sensitivity in adult mice and humans, yet the Acrp30 profile over the lifespan and its hormonal regulation in vivo have not been previously described. Hence, we set forth to determine whether hormonal and metabolic changes associated with sexual maturation, reproduction, aging, and calorie restriction affect Acrp30. In mice, Acrp30 levels increase during sexual maturation by 4-fold in males and 10-fold in females. Neonatal castration (CX) allows Acrp30 of adults to reach female levels. CX in adults does not lead to female Acrp30 levels unless glucocorticoid exposure is elevated simultaneously by implant. Ovariectomy of infant mice does not interfere with the pubertal rise of Acrp30. However, ovariectomy in adults increases Acrp30. Estrogen suppressed Acrp30 in mice and 3T3-L1 adipocytes. In parallel to changes in estrogen action, Acrp30 decreased in late gestation but increased in both calorie-restricted and old (anovulatory) mice. The reduction of Acrp30 in lactating dams is consistent with a suppressive effect of prolactin and a stimulating effect of bromocriptine. In summary, Acrp30 levels in serum are under complex hormonal control and may play a key role in determining systemic insulin sensitivity under the respective conditions. *Diabetes* 52:268–276, 2003

From the [1]Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York; the [2]Obesity Research Center, Saint Luke's-Roosevelt Hospital Center, Columbia University College of Physicians and Surgeons, New York, New York; the [3]Research Division, Joslin Diabetes Center, Boston, Massachusetts; the [4]W. Harry Feinstone Department of Molecular Microbiology & Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; the [5]Bone Morphometry Laboratory, Division of Endocrinology and Metabolism, Center for Osteoporosis and Metabolic Bone Diseases, Department of Internal Medicine, and the Central Arkansas Veterans Healthcare System, University of Arkansas for Medical Sciences, Little Rock, Arkansas; and the [6]Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, New York.

Address correspondence and reprint requests to Philipp E. Scherer, Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. E-mail: scherer@aecom.yu.edu.

Received for publication 14 July 2002 and accepted in revised form 28 October 2002.

Acrp30, adipocyte complement–related protein of 30 kDa; CR, calorie restriction; CX, castration; E2, 17β-estradiol; ER, estrogen receptor; 125I, 125Iodine; OVX, ovariectomy; Pit-1, pituitary transcription factor-1; PND, postnatal day; SSC, sodium chloride–sodium citrate.

Adipose tissue secretes several circulating proteins that appear to effect carbohydrate and lipid metabolism (1). Elevated levels of some of these factors, including tumor necrosis factor-α (2), plasminogen-activator inhibitor-1 (3), angiotensinogen (4), and the recently identified protein resistin (5), are associated with insulin resistance. On the other hand, replacement of fat-specific proteins like leptin or adipocyte complement–related protein of 30 kDa (Acrp30)/adiponectin is associated with increased insulin sensitivity (6–9). In contrast to leptin levels, which are generally proportional to overall adipose mass, Arita et al. (10) found that plasma Acrp30 levels are higher in women and lean individuals. Subsequent studies confirmed the relationship between Acrp30 levels and insulin sensitivity. For example, type 2 diabetic patients had lower Acrp30 levels than nondiabetic control subjects, and a calorie restriction (CR) program that lowered BMI by 10% increased serum Acrp30 (11). We also found significant elevations in food-restricted mice (6). In rhesus monkeys disposed to type 2 diabetes, circulating Acrp30 levels decrease before the onset of hyperglycemia (12).

Circulating Acrp30 levels may play a critical role in basal metabolism, and we therefore investigated the endocrine regulation of Acrp30 in vivo. Similar to humans, female mice have elevated plasma Acrp30 compared with males. We studied the basis for the sexually dimorphic expression of Acrp30 by assessing the gonadal contribution at various stages of development. We also studied the effects of puberty, gestation, lactation, chronic CR, and aging. The response to elevated glucocorticoid, estrogen, and prolactin exposure was studied as a first step in elucidating the endocrine mechanisms that regulate Acrp30.

RESEARCH DESIGN AND METHODS

Laboratory animals and surgical procedures. The effects of puberty, pregnancy, lactation, ovariectomy (OVX), and estrogen, prolactin, and bromocriptine implants were studied in FVB mice (Charles River Laboratories) housed in groups of two to five in filter-top cages in a controlled environment (22–25°C, 40–50% humidity, and 12-h light-dark cycle with lights on from 0600–1800). The mice had free access to water and Teklad Rodent Diet no. 8604 (Harlan Teklad) at the Association for Assessment of Laboratory Animal Care–accredited facility of the Albert Einstein College of Medicine. Avertin 1.2% (Sigma-Aldrich) was used for general anesthesia (0.2 ml/10 g body wt) to perform bilateral OVX and introduce implants. Silastic capsules of 15 mm length, 1.47 mm internal diameter, and 1.96 mm outer diameter were packed

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

with either 17β-estradiol (E2) and cholesterol (at a 1:9 ratio) or cholesterol (Steraloids). Model 1007D osmotic pumps (Alzet) delivered prolactin (Dr. A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases) and 2-bromo-α-ergocryptine (Sigma). The animal research review committee at the Albert Einstein College of Medicine approved the procedures.

The effects of neonatal and adult castration (CX) were studied in Long-Evans rats (Charles River Laboratories) housed individually in polypropylene cages covered with polyester filter bonnets in an accredited animal facility at Johns Hopkins University. The animals were maintained on a constant 16/8-h light-dark cycle with lights on at 0600 h Eastern standard time. Food and sterile tap water were available ad libitum. Female rats were bred in the laboratory, and gestational day 1 was based on the presence of a sperm plug. Pregnancy was monitored, and the day of birth constituted postnatal day (PND) 1. At PNDs 2–4, all pups were removed from the dams and held on a heating pad (with towels layered on top, to avoid overheating) to maintain body temperature. During this time, male pups assigned to have their gonads removed were anesthetized by hypothermia and castrated bilaterally, and incisions were closed with adhesive. Control males received sham operations. The procedures were approved by the Johns Hopkins animal care and use committee.

The effects of adult CX in the context of glucocorticoid treatment were studied in Swiss Webster mice (Charles River Laboratories) electronically tagged (Biomedic Data System) in plastic cages (one animal per cage) under standard laboratory conditions with a 12-h light-dark cycle at a constant temperature of 20°C and at 48% humidity. The mice had free access to water and standard Agway RMH 3000 rodent diet (Agway). Orchidectomy and prednisolone pellet implants were performed at the same time. Slow-release pellets (Innovative Research of America) of placebo or 2.1 $mg \cdot kg^{-1} \cdot day^{-1}$ prednisolone were implanted, as previously described (13). Acrp30 was measured after 28 days. The Division of Laboratory and Animal Medicine at the University of Arkansas for Medical Sciences approved the protocols.

The effects of CR and aging were studied in C57BL/6JxBALB mice (F1) maintained in a pathogen-free barrier at the Jackson Laboratories (Bar Harbor, ME). Starting at ~8 weeks of age, food intake (NIH-31 rodent diet) was measured weekly, and adjustments were made to ensure that the calorie-restricted group was allotted 60% of the ad libitum baseline intake. Mice were free of known pathogens (as detailed in quarterly reports from The Jackson Laboratory). The Jackson Laboratory animal care and use committee approved the protocol for these studies.

Blood collection. Blood samples were obtained from the tail vain or the orbital sinus by heparinized capillary tubes (Fisher) between 0900 and 1100 and stored at −20°C until measurement of Acrp30.

125Iodine (125I) labeling of Acrp30 and secondary antibody. The chloramine T method (14) with one mCi of 125I (New England Biolabs) was used to radiolabel full-length murine recombinant Acrp30 (6) for clearance studies and rabbit IgGs used for Western blots. Unincorporated label was removed by gel filtration on a PD-10 column (Amersham Pharmacia Biotech). The radiolabeled product used in the clearance studies was detected as a single band on SDS-PAGE at 30 kDa.

Cell culture. 3T3-L1 murine fibroblasts were propagated and differentiated as previously described (15). Dulbecco's modified Eagle's medium was purchased from Cellgro. E2 and ICI 182,780 were purchased from Steraloids and ICI Pharmaceuticals, respectively, whereas all other chemicals were purchased from Fisher.

Preparation of cell lysates from 3T3-L1 cells. 3T3-L1 cells were washed twice with cold PBS and lysed in TNET buffer (1% Triton X-100, 150 mmol/l NaCl, 5 mmol/l EDTA, and 50 mmol/l Tris, pH 8.0) containing 60 mmol/l octylglucoside (Roche) in the presence of protease inhibitors. Samples were centrifuged at 15,000*g* for 15 min, and the supernatant was transferred to a fresh tube. Protein determinations were made on the supernatants using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL), and equal amounts of protein were used to measure Acrp30. Monoclonal antibody-15 AER611 (Lab Vision) was used for Western blot analysis of the estrogen receptor (ER).

Total RNA isolation and Northern blot analysis. Total RNA from tissue and 3T3-L1 adipocytes was isolated with Trizol (Life Technologies). For Northern blot analysis, 20 μg of total RNA was used, as previously described (16). Blots were prehybridized in Ultrahyb (Ambion) for 2 h at 42°C. Denatured 32P-labeled DNA probes were added (2 ×10 cpm/ml), and blots were hybridized overnight (42°C). The filters were washed in 2 × sodium chloride–sodium citrate (SSC)/0.1% SDS and 0.1 × SSC/0.1% SDS before autoradiography.

Measurement of fat pad Acrp30 content. Fat pads were dissected after sacrifice, immersed in cold extraction buffer (20 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA, 10 mmol/l KCl, and 1 mmol/l phenylmethylsulfonyl fluoride), and sonicated for 20 s. The resulting homogenate was centrifuged at 4°C to separate lipids, lysate, and insoluble material. The lysate infranatant was removed and mixed with hot SDS (95°C) to a final concentration of 2% wt/vol. After 5 min, the lysates were cooled to room temperature and assayed for protein concentration by bicinchoninic acid (Pierce Protein Assay Kit; Pierce Chemical) and assayed for Acrp30 content by quantitative Western blot analysis.

Acrp30 measurements by Western blot. Acrp30 in plasma (3 μl) or tissue (50 μg protein) was measured by Western blot analysis. After SDS-PAGE (12%), proteins were transferred to BA83 nitrocellulose (Schleicher & Schuell). The blots were exposed to 125I-labeled rabbit antibody against murine Acrp30 and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), using ImageQuant 1.2 software. Each gel contained four standards of purified mouse Acrp30 at four different concentrations to ensure linearity and reproducibility of the signal. Intra- and interassay variations for Acrp30 were <10%, and the limit of detection was ~5 ng of Acrp30 (17).

Statistical analysis. The results are shown as the means ± SE. Statistical analysis was performed by one- or two-way ANOVA, unless otherwise indicated. Significance was accepted at $P < 0.05$.

RESULTS

At 1 week of age, circulating Acrp30 values in male and female FVB mice were 3.0 ± 0.8 μg/ml. By 6 weeks, Acrp30 reached 10.2 ± 2.0 and 30.1 ± 4.2 μg/ml in males and females, respectively ($P < 0.001$) (Fig. 1*A*). The clearance of intravenous [125I]Acrp30 did not differ between adult males and females (Fig. 1*B*). A number of studies have linked elevated Acrp30 levels with improved insulin sensitivity (6). Elevated serum Acrp30 levels in females are consistent with the improved insulin sensitivity of female FVB mice compared with their age-matched male counterparts, as judged by decreased glucose and insulin levels (Fig. 2*A* and *B*), as well as an improved insulin tolerance test (Fig. 2*C*). We measured both Acrp30 mRNA and protein levels in various fat pads isolated from male and female mice. Surprisingly, Northern blot analysis did not reveal significant sex differences in Acrp30 mRNA of gonadal white and interscapular brown adipose tissue (Fig. 3*A*). In contrast, Western blot analyses showed that Acrp30 protein levels within the fat depots are significantly lower in males than females, suggesting sexually dimorphic protein production and secretion in adipocytes (Fig. 3*B*).

Elevated neonatal androgen exposure is associated with sex-specific imprinting. Adult Acrp30 levels were therefore measured in Long-Evans rats that had undergone CX 2–4 days after birth. Neonatal CX allowed plasma Acrp30 in adult males to rise above controls ($P < 0.001$) and reach female values. CX in adult rats had no affect (Fig. 4*A*). Acrp30 levels in Swiss Webster and FVB mice were also unaffected by adult CX (Fig. 4*B* and data not shown). However, Acrp30 was significantly upregulated ($P < 0.001$) in an osteoporosis model that combined CX with the synthetic glucocorticoid analog prednisolone (13,18) (Fig. 4*B*). Secondary hypogonadism and increased glucocorticoid activity are also found under other physiological conditions, such as chronic food restriction. Accordingly, Acrp30 levels were higher in both young and old male F1 (C57BLxBALB) mice under chronic calorie-restriction than in animals with ad libidum access to food (28.2 + 2.0 and 30.0 + 2.0 μg/ml vs. 17.0 + 2.0 and 18.0 + 2.0 μg/ml) (Fig. 4*C*). In agreement with observations in Fig. 3, changes in serum Acrp30 are brought about post-transcriptionally, since mRNA levels in gonadal white adipose tissue remain unchanged (Fig. 4*D*).

To determine whether ovarian maturation during puberty plays a role in the sex-specific expression of Acrp30

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

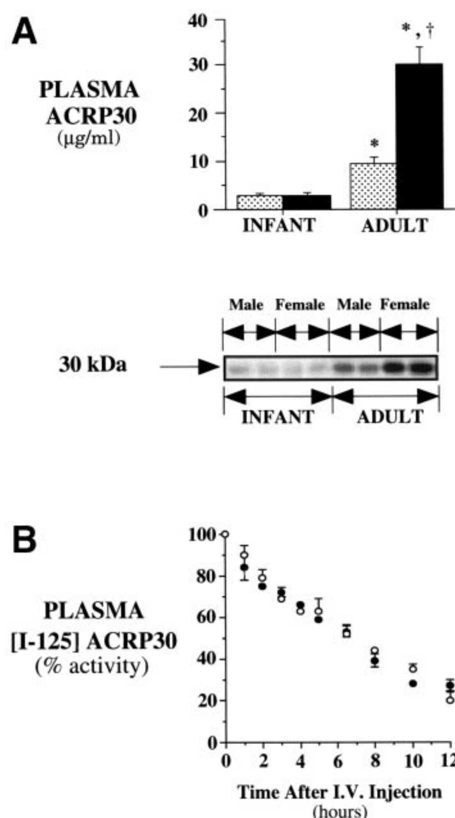

FIG. 1. *A*: Male (▨; $n = 5$) and female (■; $n = 5$) circulating Acrp30 levels in 7-day-old infant and 40-day-old adult FVB mice. Plasma samples were collected from the tail vein of male and female mice and analyzed for Acrp30 by Western blot analysis using rabbit antisera against murine Acrp30 and [I-125]-labeled anti-rabbit IgGs. Each gel contained four standards of purified mouse Acrp30 at four different concentrations to generate absolute values and ensure linearity and reproducibility of the signal. Two-way ANOVA using age and sex as independent variables identified significant ($P < 0.05$) effects of age in males and females as well as a significant effect of sex between adults. Representative Western bands are shown below the bar graph. *B*: Clearance of [I-125]Acrp30 in adult male (○; $n = 3$) and female (●; $n = 3$) FVB mice. [I-125]-labeled full-length murine recombinant Acrp30 was injected intravenously, and blood was sampled at the indicated time points to measure the levels of γ-radiation. Two-way ANOVA did not identify any significant differences, using sex and time after injection as independent variables. I.V., intravenous. *Significant ($P < 0.05$) effect of age; †significant ($P < 0.05$) effect of sex.

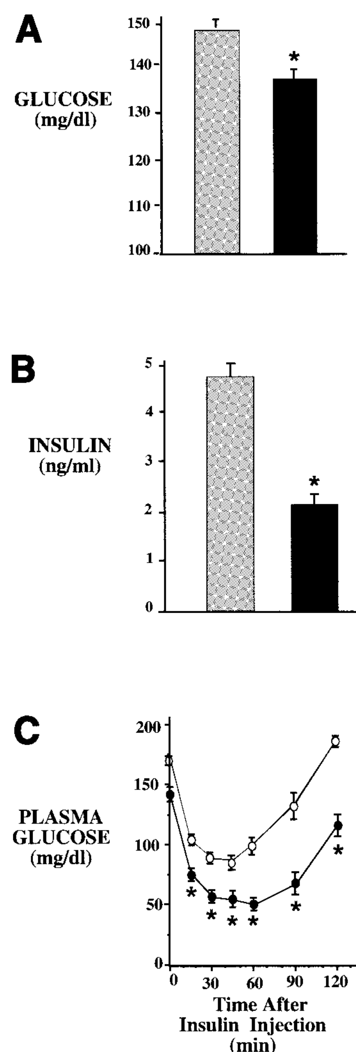

FIG. 2. Increased insulin sensitivity in female FVB mice. Plasma glucose (*A*) and plasma insulin levels (*B*) were measured in young male and female FVB mice. *C*: Insulin tolerance test in young male and female FVB mice. Recombinant insulin at equal amounts per body weight were injected (0.5 units/kg body wt) and glucose was measured at the designated time points. Two-way ANOVA using sex, glucose, and time as independent variables identified significant ($P < 0.05$) effects of sex. *Significant ($P < 0.05$) effects of sex.

in females, the ovaries were surgically removed in FVB mice at 1 week of age. OVX before puberty (1 week) did not prevent Acrp30 from reaching normal female levels (Fig. 5*A*). In fact, OVX in adults allowed plasma Acrp30 to increase 10 days after surgery above sham-OVX controls (42.1 ± 3.2 vs. 30.5 ± 4.2 μg/ml, respectively, in OVX and sham-OVX; $P < 0.05$). The effect of estrogen withdrawal associated with OVX was reflected in the uterine weights (18 ± 4 and 98 ± 10 mg, respectively, in OVX and sham-OVX; $P < 0.001$). Mice that received E2 implants and OVX were examined to determine whether estrogen might suppress Acrp30. In agreement with a suppressive effect of estrogen, Acrp30 levels decreased after 10 days of continuous exposure to supraphysiological levels of E2 (250–350 pg/ml) (Fig. 5*A*). Once again, uterine weights reflected the bioactivity of the E2 implants (92.4 ± 8.3 and 21.9 ± 4.0 mg, respectively, in E2- and vehicle-implanted mice).

This suggests that estrogen exerts a negative impact on Acrp30 levels in vivo. Because estrogen levels drop dramatically after ovarian cessation, we predicted that levels of Acrp30 should increase in old anovulatory mice. To test this hypothesis, we performed a cross-sectional study, looking at age-related differences in serum Acrp30 levels in young (4–5 months) and old (24–30 months) F1 (C57BLxBALB) mice (Fig. 5*B*). Acrp30 levels are ~80% higher in old mice. We cannot, of course, exclude other age-related influences on Acrp30 expression. However, if the increase in Acrp30 under food restriction is a consequence of secondary hypogonadism, we would expect to see a rise of Acrp30 in response to food restriction in young mice (reduced estrogen in response to reduced caloric intake) but not in old anovulatory mice (low estrogen under all conditions), and this is indeed the case (Fig. 5*B*). Similar to the observations in Figs. 3 and 4, changes in serum Acrp30 are brought about posttranscriptionally, since mRNA levels in gonadal white adipose tissue remain unchanged (Fig. 5*C*). This observation emphasizes that depending on the physiological state, tissue mRNA and serum protein levels do not always correlate.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

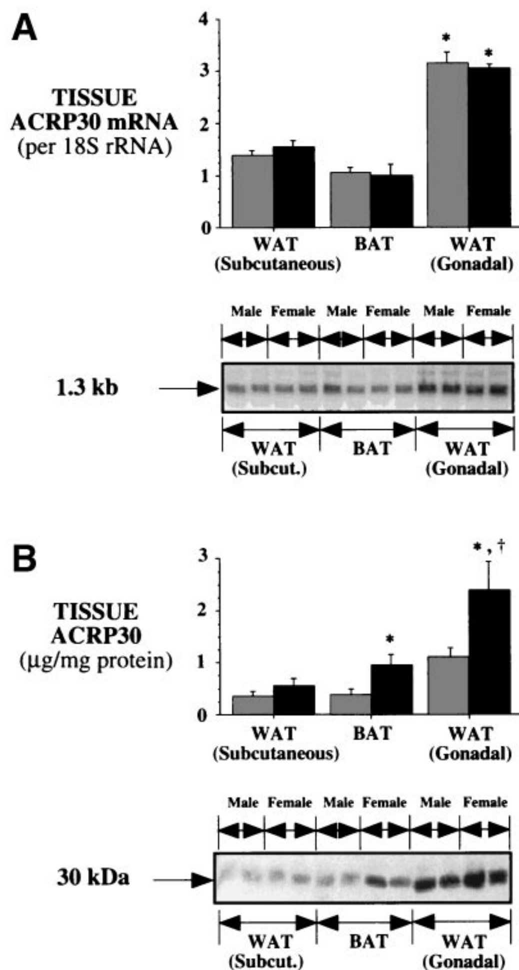

FIG. 3. ***A*: Acrp30 mRNA levels in adipose tissue in adult male (▨; $n = 3$) and female (■; $n = 3$) FVB mice. Total RNA was extracted from dorsal subcutaneous white adipose tissue (WAT), interscapular brown adipose tissue (BAT), and epididymal or perimetrial/ovarian white adipose tissue and analyzed for Acrp30 mRNA by Northern blot. Two-way ANOVA using fat depot and sex as independent variables identified a significant ($P < 0.05$) effect of fat depot in males and females but no effect of sex. Representative 1.2-kb Northern bands are shown below the bar graph. Subcut., subcutaneous. *Significant ($P < 0.05$) effect of fat depot. *B*: Intracellular Acrp30 levels in adipose tissue from adult (▨; $n = 3$) and female (■; $n = 3$) FVB mice. Equal amounts of protein (50 μg) from various fat depots were analyzed for Acrp30 by Western blot analysis using rabbit antisera against murine Acrp30 and [I-125]-labeled anti-rabbit IgGs. Each gel contained four standards of purified mouse Acrp30 at four different concentrations to generate absolute values and ensure linearity and reproducibility of the signal. Two-way ANOVA using sex and fat depots as independent variables identified significant ($P < 0.05$) effects of sex in each depot as well as a significant effect of depot in the case of gonadal WAT. A representative Western blot is shown below the bar graph. Subcut., subcutaneous. *Significant ($P < 0.05$) effect of sex; †significant ($P < 0.05$) effect of depot.**

The suppression of Acrp30 in vivo may, however, be an indirect effect of elevated E2 caused by a number of downstream effects, such as the estrogen-mediated suppression of pituitary leuteinizing hormone and follicle-stimulating hormone. Hence, 3T3-L1 adipocytes were used to determine whether the effects of estrogen in vivo were direct or indirect. FCS (10%) used to maintain adipocyte cultures is already rich in gestational estrogens, but the addition of increasing amounts of E2 suppressed Acrp30 levels (Fig. 6*A*; left panel), and the effect was blocked by the potent ER antagonist ICI 182,780 (19). Secretion of the protein into the extracellular medium was blocked as well (Fig. 6*A*; right panel). The specificity of estrogen action is supported by the presence of ER in differentiated 3T3-L1 adipocytes detected with a monoclonal antibody against murine ERα (Fig. 6*B*). A similar 66-kDa band was also detected in uterine extracts without immunoprecipitation. Interestingly, treatment of 3T3-L1 adipocytes with E2 suppressed Acrp30 mRNA levels, suggesting that in this isolated in vitro system, mRNA and protein go hand in hand (Fig. 6*C*). This effect reflects direct action of the ER on the Acrp30 gene, since similar effects can be observed in the presence of the protein biosynthesis inhibitor cycloheximide. Pregnancy is associated with a remarkable increase of various estrogens (20), and consistent with the E2 effects described herein, Acrp30 in mice decreased in the second and third week of gestation ($P < 0.05$) (Fig. 7*A*). Food intake increases by nearly 100% late in gestation, but the suppression of plasma Acrp30 late in gestation was evident even after 1 week of 50% CR ($P < 0.05$) (Fig. 7*B*). Despite the postpartum drop in estrogen, Acrp30 levels remained suppressed during lactation ($P < 0.05$) (Fig. 7*A*).

The effect of elevated lactogens seen during lactation and late gestation was assessed with osmotic minipumps releasing 50–100 μg of prolactin per day. Consistent with a suppressive effect of prolactin, circulating Acrp30 levels decreased after 72 h of elevated prolactin exposure ($P < 0.05$) (Fig. 8*A*). In contrast, Acrp30 levels increased with bromocriptine implants, presumably under dopaminergic inhibition of prolactin synthesis and secretion ($P < 0.05$) (Fig. 8*A*).

The correlation between low body weight, low prolactin, elevated insulin sensitivity, and high Acrp30 levels seen with CR is also evident in the Snell dwarf mouse (Fig. 8*B*). This model is homozygous for a mutation in pituitary transcription factor-1 (Pit-1), which plays a key role in the pituitary's expression of prolactin, growth hormone, and thyroid-stimulating hormone (21).

DISCUSSION

We observed that circulating Acrp30 levels in mice increased abruptly by the third week of postnatal life but were relatively stable thereafter. In contrast to Acrp30, circulating levels of another adipose-specific marker, leptin, are relatively constant from 1 to 6 weeks of age and only begin to rise by the seventh week (22). What triggers the rise of Acrp30 early in puberty is not known. Our results, however, indicate that it does not involve the maturation of a gonadal feedback loop because surgical excision of the testes or ovaries before puberty did not interfere with the rise of Acrp30 in adults. Marked differences between the peripubertal secretions of male and female gonads support the likelihood that the pubertal Acrp30 surge is mediated by nongonadal mechanisms, and the similar timing in the male and female Acrp30 surge suggests a common mechanism. Previous studies suggest that the peripubertal surges of growth hormone, IFG-I, prolactin, and luteinizing hormone are also triggered by nongonadal mechanisms (23–25).

Adult female mice have higher Acrp30 levels than males, in agreement with the sexual dimorphism previously de-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

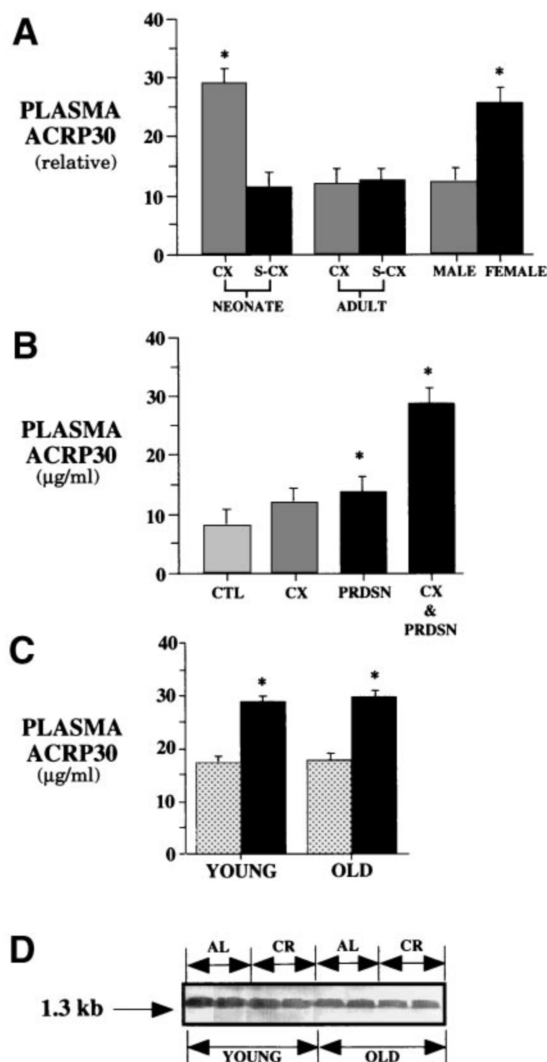

FIG. 4. ***A*****: The effects of neonatal CX on Acrp30 levels in adult male Long-Evans rats. Blood was sampled from adult male rats (100 days old) that were either intact (Male; body weight = 488 ± 20, n = 10), castrated at 2–4 days after birth (CX-Neonate; body weight = 379 ± 9, n = 10), or castrated at 60–70 days after birth (CX-Adult; body weight = 436 ± 19, n = 10). Plasma samples from sham-operated animals (S-CX–Neonate and S-CX–Adult) and ovariectomized female (Female) rats were included for comparison. Plasma was analyzed for Acrp30 by Western blot analysis as previously described. Plasma leptin values were 5.5 ± 0.7 (Male), 9.2 ± 0.9 (CX-Neonate), 4.7 ± 0.5 (CX-Adult), and 7.6 ± 0.8 ng/ml (Female), respectively. One-way ANOVA using effect of treatment as the independent variable identified a significant (P < 0.05) effect of neonatal CX. The effect of sex was not significant between adult females and males that underwent neonatal CX. *Significant (P < 0.05) effect of neonatal CX.** ***B*****: The effects of CX and glucocorticoid treatment on Acrp30 levels. Mice were 6 months old at the start of the experiment. CX and prednisolone (PRDSN) pellet implantation were performed at the same time. Slow-release pellets of 2.1 mg · kg^{-1} · day^{-1} prednisolone were implanted, and blood was sampled for Acrp30 28 days later. Blood was sampled from intact mice (CTL; n = 16), castrated (CX; n = 8), prednisolone-treated (PRDSN; n = 13) and the combination of CX and prednisolone-treatment (CX & PRDSN; n = 11). One-way ANOVA using effect of treatment as the independent variable identified significant (P < 0.05) effects of prednisolone treatment as well as the combination of prednisolone treatment and CX. *Significant (P < 0.05) effects of prednisolone treatment as well as the combination of prednisolone treatment and CX.** ***C*****: Effects of CR and aging in male mice. Blood samples were obtained for Acrp30 measurement at 5 months (YOUNG; n = 5) and after 24–30 months (OLD; n = 5). F1 mice (C57BLxBALB) used here were maintained at The Jackson Laboratories. At 2 months after birth, calorie-restricted mice (■) started receiving 60% of the baseline intake of the ad libitum–fed group (light bars). Feed intake and spillage were measured routinely for all mice, and adjustments in feed allotments were made to ensure that calorie-restricted mice received 60% of their individual baseline intake. Blood samples were obtained for Acrp30 measurement at 5 months (YOUNG) and after 24–30 months (OLD). Two-way ANOVA using effects of CR and age as the independent variables identified a significant (P < 0.05) effect of calorie-restriction. *Significant (P < 0.05) effect of calorie-restriction.** ***D*****: Effects of caloric-restriction and aging on male Acrp30 mRNA levels. Gonadal fat pads from the mice used in panel *C* were used for RNA isolation and Northern blot analysis. mRNA levels for Acrp30 remain unchanged upon normalization with a β-actin probe. AL, ad libitum–fed.**

scribed in humans (10,11,26). A similar sexual dimorphism has also been described for leptin, even after accounting for differences in adipose tissue mass (27). Like humans and mice, the Long-Evans rat exhibits a sex-specific difference in plasma Acrp30 as well as leptin. We demonstrate for the first time that neonatal CX clearly feminized both Acrp30 and leptin levels in the adult male. It is unlikely that the lack of androgens during the neonatal period directly affects Acrp30 levels. Much rather, the lack of androgens during this critical postnatal period exerts its effect through an imprinting mechanism presumably at the level of the brain or adipose tissue. In either mouse or rat, adult CX did not have a significant effect on Acrp30. Hence, the testes may only have an irreversible effect in the sexual differentiation of adipose tissue during the critical neonatal period, as previously shown in the hypothalamus, pituitary, and liver (28–30). We observed that male fat depots had lower Acrp30 levels, even though Acrp30 mRNA levels were indistinguishable between males and females, indicative of a sexual dimorphism with respect to posttranscriptional regulation. We report similar discrepancies between mRNA and serum protein levels under two additional conditions in vivo. This emphasizes the need to measure serum protein levels as the final readout and highlights the dangers of drawing any conclusions based on tissue mRNA levels alone. However, in many instances, mRNA and protein levels do agree with each other, and we cannot draw the conclusion from these results that serum Acrp30 levels are exclusively governed by posttranscriptional mechanisms.

Mature ovaries have a mild but significant influence on adult Acrp30 levels. OVX in young cycling mice induced plasma Acrp30 (10 days after surgery), and E2 implants reversed the effect. Increased adiposity is generally associated with OVX. However, the increase of Acrp30 in this case was measured before weight gain. The ability to block the effect of E2 in 3T3-L1 adipocytes with ICI 182,780, an ER antagonist, suggests that E2 acts directly on adipose tissue. However, whereas 3T3-L1 adipocyte mRNA and protein levels correlate, such correlations were not observed in the in vivo setting. The suppression of Acrp30 we observed during pregnancy and the elevation of Acrp30 in anovulatory mice aged 24–30 months are consistent with opposite extremes of estrogen action. The parallel increase of adipose mass, aromatase activity, and nonovarian estrogens may also play a key role in the suppression of plasma Acrp30 in obese individuals (31). The correlation during puberty between increasing estrogen and Acrp30 is therefore distinct from their inverse relationship in adulthood. However, more rigorous testing is needed to determine the physiological significance of estrogen action on Acrp30.

Do plasma Acrp30 levels thus far reflect the insulin-sensitizing effects of recombinant Acrp30 and all of the

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

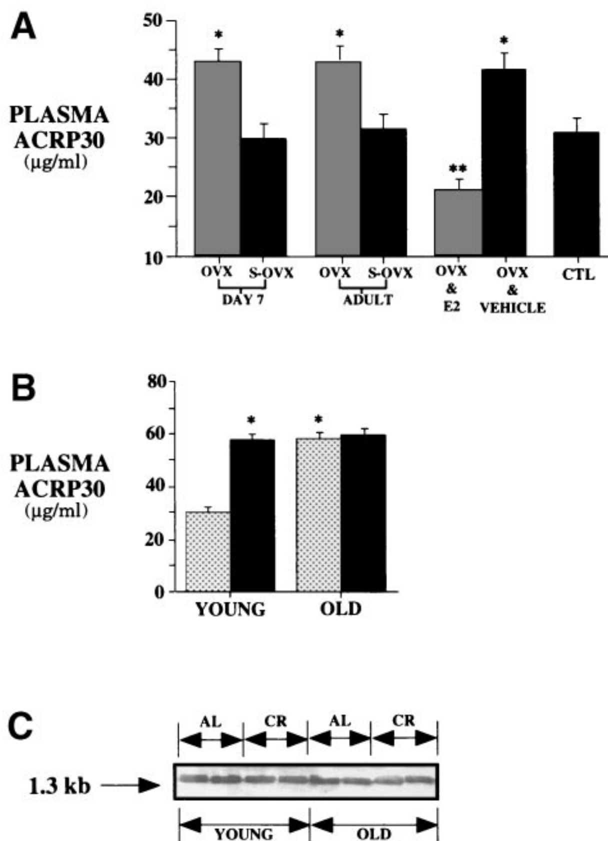

FIG. 5. *A*: Effects of OVX and elevated estrogen exposure on plasma Acrp30. Blood was sampled from adult FVB mice at 55 days of age that were intact (CTL; $n = 5$), mice that underwent bilateral OVX 7 days after birth or were sham operated (SOVX; $n = 5$ each), mice that underwent bilateral OVX at 45 days old (ADULTS) or were sham operated (S-OVX; $n = 5$ each), and adult mice that underwent a combination of bilateral OVX and E2 implants (OVX & E2) or vehicle ($n = 5$ each). One-way ANOVA using effect of treatment as the independent variable identified significant ($P < 0.05$) effects of bilateral OVX during infancy and adulthood as well as the combination of bilateral OVX and E2 treatment. *Significant ($P < 0.05$) effects of bilateral OVX during infancy and adulthood; **significant ($P < 0.05$) effects of the combination of bilateral OVX and E2 treatment. *B*: Effects of CR and aging in female mice. Blood samples were obtained for Acrp30 measurement at 5 months (YOUNG; $n = 5$) and after 24–30 months (OLD; $n = 5$). F1 mice (C57BLxBALB) used here were maintained at The Jackson Laboratories. At 2 months after birth, calorie-restricted mice (■) started receiving 60% of the baseline intake of the ad libitum–fed group (light bars). Feed intake and spillage were measured routinely for all mice, and adjustments in feed allotments were made to ensure that calorie-restricted mice received 60% of their individual baseline intake. Blood samples were obtained for Acrp30 measurement at 5 months (YOUNG) and after 24–30 months (OLD). Two-way ANOVA using effects of CR and age as the independent variables identified a significant ($P < 0.05$) effect of CR only in young females. *Significant ($P < 0.05$) effect of CR. *C*: Effects of CR and aging on female Acrp30 mRNA levels. Gonadal fat pads from the mice used in *B* were used for RNA isolation and Northern blot analysis. mRNA levels for Acrp30 remain unchanged upon normalization with a β-actin probe. AL, ad libitum–fed.

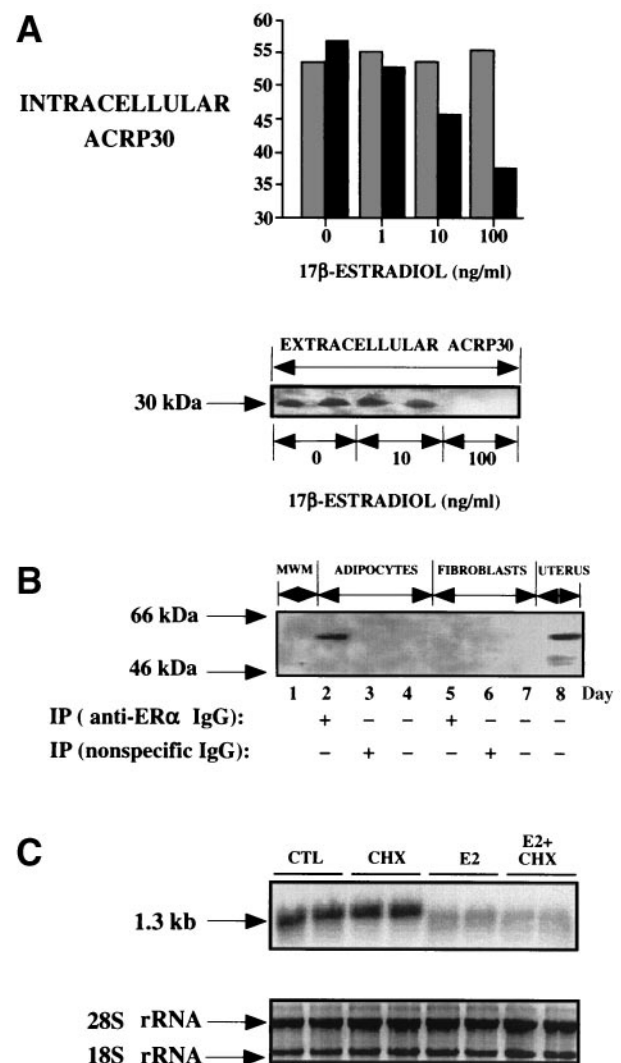

FIG. 6. *A*: Acrp30 levels in 3T3-L1 adipocytes after overnight E2 exposure. Top panel: Intracellular Acrp30 levels in 3T3-L1 adipocytes after overnight E2 exposure in the presence (▒) or absence (■) of the ER antagonist (ICI 182,780). Differentiated 3T3-L1 adipocytes (day 8) were exposed to 10% FCS containing 0, 1, 10, and 100 ng/ml of E2 with or without 100 nmol/l ICI 182,780 for 12 h. Cells were then washed twice with cold PBS and lysed in TNET buffer. Equivalent amounts (50 μg) of total protein extract were analyzed by Western blot analysis as previously described. Numbers on the *y*-axis represent arbitrary units. Bottom panel: Extracellular Acrp30 levels. Cells were treated as described in the top panel; after the 12-h treatment, the medium was replaced with serum-free Dulbecco's modified Eagle's medium, and cells were allowed to secrete for an additional 3 h in the continued presence of the indicated E2 concentrations. The medium was subsequently analyzed by Western blot analysis for the levels of Acrp30. *B*: ER expression in differentiated adipocytes (day 8), confluent murine 3T3-L1 preadipocytes (day 0), and uterus. Cell lysates were incubated with protein A-Sepharose or rabbit anti-murine ER immune or preimmune sera. Extracts were analyzed by enhanced chemiluminescence on 10% SDS-PAGE for ER using a mouse monoclonal antibody. Total uterine protein extract was included as a positive control. MWM, molecular weight markers. *C*: Estrogen directly represses Acrp30 levels in 3T3-L1 adipocytes. Mature (day 8) 3T3-L1 adipocytes were incubated for 12 h in either the presence or absence of 100 ng/ml of E2 and 300 μmol/l cycloheximide (CHX), as indicated. Total RNA was subsequently extracted and analyzed by Northern blot analysis with a probe for mouse Acrp30. 18S and 28S RNA were visualized on a separate gel to ensure equal loading. CTL, control.

correlative data that illustrate the relationship between insulin resistance and low plasma Acrp30? Changes in insulin action during puberty have been poorly described in rodent models. We showed that in young adult mice, the fasting glucose, insulin, and insulin tolerance tests indicate that females are more insulin sensitive than their male littermates. Early studies in adult rats showed that 95% pancreatectomy resulted in a higher incidence of diabetes in males than females even after CX or OVX (32). The development of diabetes after pancreatectomy did not differ between female rats that were masculinized by neonatal exposure to elevated testosterone and male rats (33). A sexual dimorphism has also been described for

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

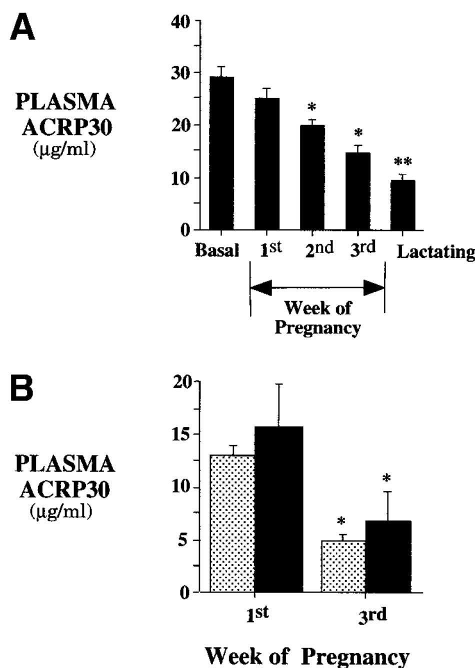

FIG. 7. *A*: Circulating Acrp30 levels during gestation and lactation. Serial blood samples were obtained from young female FVB ($n = 5$) mice before mating; during the first, second, or third trimesters of pregnancy; and after 1 week of lactation. One-way ANOVA using the effect of the individual stage of reproduction as the independent variable identified significant ($P < 0.05$) effects in the second and third trimesters of pregnancy. *Significant ($P < 0.05$) effects. **Significant effect evident with 1 week of lactating compared with the last trimester of pregnancy. *B*: Circulating Acrp30 levels during gestation in response to CR. Samples were obtained from tail vein in the morning of days 12.5 and 18.5 of pregnancy in C57BL mice. On day 12.5, animals were separated into two groups, with one group receiving ~50% (■; $n = 6$) of the calories consumed by the ad libitum–fed group (light bars; $n = 6$). Two-way ANOVA using effects of CR and stage of pregnancy as the independent variables identified a significant ($P < 0.05$) effect of pregnancy but not CR. *Significant ($P < 0.05$) effect of pregnancy.

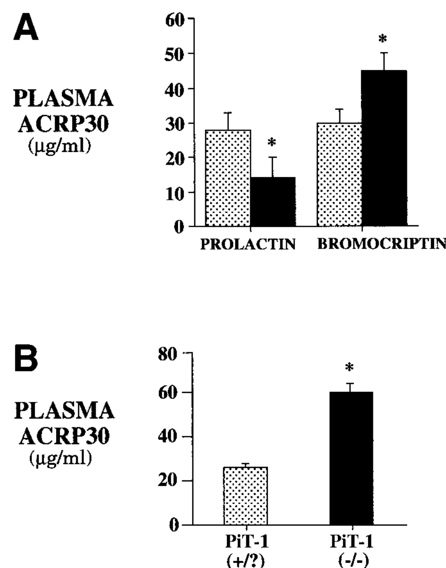

FIG. 8. *A*: Effects of elevated prolactin and bromocriptin exposure on circulating Acrp30. Equilibrated micro-osmotic pumps containing either prolactin or the dopamine agonist bromocriptine were placed in the dorsal subcutaneous area of mature female C57BL/6J mice ($n = 4$ per group). The rate of release of prolactin and bromocriptine were 50–100 μg/day and 100–150 μg/day, respectively. Blood samples were obtained before (light bars) and 72-h after (■) continuous hormone exposure. Two-way ANOVA identified a significant ($P < 0.05$) effect of both treatment and time after treatment. *Significant ($P < 0.05$) effect of both treatment and time after treatment. *B*: Circulating Acrp30 in Pit-1 (−/−) mice. Blood was sampled from young (4-month-old) female Snell dwarf mice (■; −/−, body weight = 10.9 + 1.1 g, $n = 5$) and their litter mate controls (light bar; +/+ or +/−, body weight = 25.7 + 2.3 g, $n = 5$) and analyzed for Acrp30 as previously described. Nonparametric two-sample t test indicated a significant difference between groups ($P < 0.05$). *Significant difference ($P < 0.05$) between groups.

non–insulin-dependent diabetes in the Wistar fatty rat on a high-sucrose diet (34).

Both humans and rodents develop insulin resistance during pregnancy (35,36). Here, we show for the first time that Acrp30 levels are suppressed from mid-gestation until weaning. Of all the endocrine changes that peak around pregnancy, placental lactogens and prolactin best fit the time course for the suppression of Acrp30. Placental prolactin-like molecules appear in the maternal circulation around mid-gestation, whereas pituitary prolactin levels increase progressively during gestation and remain elevated until suckling ceases (37). Isolated hyperprolactinemia is associated with insulin resistance, which is sometimes treated with various dopamine receptor agonists like bromocriptine (38,39). Indeed, the expected effects of prolactin and bromocriptine implants were observed on plasma Acrp30 in adult mice. Once again, the parallel rise of prolactin and Acrp30 in plasma seen during puberty should be distinguished from their apparently inverse relationship in adulthood.

It is well known that chronic CR (not malnutrition) increases insulin sensitivity in adult rodents compared with ad libitum feeding (40). Based on the consistent correlation between plasma Acrp30 and insulin sensitivity, it was not surprising to see that CR is associated with elevated plasma Acrp30 in young male and female mice. CR in rodents has also been used to understand the process of aging since the life-extending effects of food restriction were first in described rats (41). One theory is that CR delays the onset of changes associated with senescence, and we tested this by measuring Acrp30 levels in young and old ad libitum–fed and CR mice. The progression of biological senescence in common strains of laboratory mice is well advanced by 23–30 months, yet ad libitum–fed males maintained the same levels of Acrp30 as seen at 3–5 months. Moreover, CR led to a similar induction of Acrp30 in young and old males. On the other hand, old female mice had higher Acrp30 levels than young mice, and CR failed to stimulate Acrp30. The induction of Acrp30 we observed with the combination of CX and prednisolone treatment in Swiss Webster mice may be related to the mild stress response and secondary hypogonadism triggered by CR (42,43).

Because of a mutation in Pit-1, the Snell dwarf mouse (−/−) is a genetic model of anterior pituitary hormone deficiency of prolactin, thyroid-stimulating hormone, and growth hormone (42,44,45). We observed a significant elevation of plasma Acrp30 in the Snell dwarf mouse that is consistent with its marked elevation in insulin sensitivity (46,47). Elevated insulin sensitivity is considered to play a key role in life extension across phyla (48).

The evidence for a role of Acrp30 in insulin sensitivity was recently underlined by the development of mice carrying a chromosomal deletion of Acrp30. Thus far, two independent reports describe that in oral glucose tolerance tests and insulin tolerance tests, the Acrp30 (+/−)

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

and Acrp30 (−/−) mice have decreased insulin sensitivity (49,50). Future studies may compare the insulin sensitivity between male and female Acrp30 (−/−) mice to determine the significance of Acrp30 in sex-specific differences or CR.

How is it possible that a female steroid hormone like estrogen could suppress Acrp30 if basal Acrp30 values are clearly greater in females than males? This paradox suggests that the inhibitory effect of elevated estrogen exposure in females cannot eliminate the sex-specific "set point." We showed that early postnatal CX is the only manipulation that could alter the sexual dimorphism, suggesting that neonatal testicular secretions play a major role in establishing a sex-specific set point for Acrp30. Therefore, we propose that the set point, which determines basal Acrp30 levels in males and females, is established by the neonatal testosterone surge. Estrogen in adults only modulates Acrp30 values.

ACKNOWLEDGMENTS

This work was supported by a Hormones/Membrane Interactions Training Grant from the National Institutes of Health (T32 DK 07513-15; to T.P.C.), the University of California at Davis, a National Research Service Award (F32 DK61228; to T.P.C.), an American Diabetes Medical Scientist Training Grant (to A.H.B.), an American Diabetes Association Research Grant Award (to M.E.P.), the Adler Foundation (M.E.P. and J.C.J.-C.), a National Institutes of Health Medical Scientist Training Grant (T32-GM07288; to P.I.), a VA Research Enhancement Award Program Grant and VA Merit Review Grant from the Office of Research and Development, Department of Veterans Affairs, and National Institutes of Health grants R01-AR46191 (to R.S.W.) and 1R01-DK55758 (to P.E.S.).

We are thankful to the members of Dr. Scherer's laboratory for their critical comments on the manuscript, Drs. K. Flurkey and D. Harrison for kindly providing serum samples from Snell dwarf mice (The Jackson Laboratory), Dr. B. Frenkel for initiating the glucocorticoid studies (USC Institute for Genetic Medicine), Dr. A.F. Parlow for prolactin (National Hormone and Peptide Program, NIDDK) and Drs. P. Wise (University of California at Davis) and A. Etgen (Albert Einstein College of Medicine) for their helpful suggestions.

REFERENCES

1. Havel PJ: Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. *Curr Opin Lipidol* 13:51–59, 2002
2. Hotamisligil GS: The role of TNFalpha and TNF receptors in obesity and insulin resistance. *J Intern Med* 245:621–625, 1999
3. Loskutoff DJ, Samad F: The adipocyte and hemostatic balance in obesity: studies of PAI-1. *Arterioscler Thromb Vasc Biol* 18:1–6, 1998
4. Karlsson C, Lindell K, Ottosson M, Sjostrom L, Carlsson B, Carlsson LM: Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to angiotensin II. *J Clin Endocrinol Metab* 83:3925–3929, 1998
5. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA: The hormone resistin links obesity to diabetes. *Nature* 409:307–312, 2001
6. Berg AH, Combs T, Du X, Brownlee M, Scherer PE: The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 7:947–953, 2001
7. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF: Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 98:2005–2010, 2001
8. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941–946, 2001
9. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL: Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401:73–76, 1999
10. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79–83, 1999
11. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 20:1595–1599, 2000
12. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, Matsuzawa Y: Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* 50:1126–1133, 2001
13. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC: Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids: potential mechanisms of their deleterious effects on bone. *J Clin Invest* 102:274–282, 1998
14. Harlow E, Lane D: *Antibodies: A Laboratory Manual*. Plainview, NY, Cold Spring Harbor Laboratory Press, 1988
15. Engelman JA, Lisanti MP, Scherer PE: Specific inhibitors of p38 MAP kinase block 3T3–L1 adipogenesis. *J Biol Chem* 273:32111–32120, 1998
16. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270:26746–26749, 1995
17. Combs TP, Wagner JA, Berger J, Doebber T, Wang W-J, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DS, Chatterjee K, Weiss S, Larson PJ, Gottesdiener KM, Gertz BG, Charron MJ, Scherer PE, Moller DE: Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* 143:998–1007, 2002
18. Frey FJ: Kinetics and dynamics of prednisolone. *Endocr Rev* 8:453–473, 1987
19. Wakeling AE, Bowler J: ICI 182,780, a new antioestrogen with clinical potential. *J Steroid Biochem Mol Biol* 43:173–177, 1992
20. Branch DW: Physiologic adaptations of pregnancy. *Am J Reprod Immunol* 28:120–122, 1992
21. Li S, Crenshaw EB 3rd, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG: Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* 347:528–533, 1990
22. Cheung CC, Thornton JE, Nurani SD, Clifton DK, Steiner RA: A reassessment of leptin's role in triggering the onset of puberty in the rat and mouse. *Neuroendocrinology* 74:12–21, 2001
23. Jansson JO, Ekberg S, Isaksson OG, Eden S: Influence of gonadal steroids on age and sex-related secretory patterns of growth hormone in the rat. *Endocrinology* 114:1287–1294, 1984
24. Handelsman DJ, Spaliviero JA, Scott CD, Baxter RC: Hormonal regulation of the peripubertal surge of insulin-like growth factor-I in the rat. *Endocrinology* 120:491–496, 1987
25. Urbanski HF, Ojeda SR: The development of afternoon minisurges of lutenizing hormone secretion in prepubertal female rats is ovary dependent. *Endocrinology* 118:1187–1193, 1986
26. Yang WS, Jeng CY, Wu TJ, Tanaka S, Funahashi T, Matsuzawa Y, Wang JP, Chen CL, Tai TY, Chuang LM: Synthetic peroxisome proliferator-activated receptor-gamma agonist, rosiglitazone, increases plasma levels of adiponectin in type 2 diabetic patients. *Diabetes Care* 25:376–380, 2002
27. Rosenbaum M, Pietrobelli A, Vasselli JR, Heymsfield SB, Leibel RL: Sexual dimorphism in circulating leptin concentrations is not accounted for by differences in adipose tissue distribution. *Int J Obes Relat Metab Disord* 25:1365–1371, 2001
28. Jansson JO, Frohman LA: Differential effects of neonatal and adult androgen exposure on the growth hormone secretory pattern in male rats. *Endocrinology* 120:1551–1557, 1987
29. Duncan JA, Dalkin AC, Barkan A, Regiani S, Marshall JC: Gonadal

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024

regulation of pituitary gonadotropin-releasing hormone receptors during sexual maturation in the rat. *Endocrinology* 113:2238–2246, 1983
30. Gustafsson JA, Ingelman-Sundberg M, Stenberg A: Neonatal androgenic programming of hepatic steroid metabolism in rats. *J Steroid Biochem* 6:643–649, 1975
31. Zumoff B: Hormonal abnormalities in obesity. *Acta Med Scand Suppl* 723:153–160, 1988
32. Foglia VG, Schuster N, Rodriguez RR: Sex and diabetes. *Endocrinology* 41:428–433, 1947
33. Foglia VG, Basabe JC, Chieri RA: Evolution of diabetes after early testosterone treatment in rats. *Diabetologia* 5:258–259, 1969
34. Kava RA, West DB, Lukasik VA, Wypijewski C, Wojnar Z, Johnson PR, Greenwood MR: The effects of gonadectomy on glucose tolerance of genetically obese (fa/fa) rats: influence of sex and genetic background. *Int J Obes Relat Metab Disord* 16:103–111, 1992
35. Ryan EA, O'Sullivan MJ, Skyler JS: Insulin action during pregnancy: studies with the euglycemic clamp technique. *Diabetes* 34:380–389, 1985
36. Kuhl C: Glucose metabolism during and after pregnancy in normal and gestational diabetic women. 1. Influence of normal pregnancy on serum glucose and insulin concentration during basal fasting conditions and after a challenge with glucose. *Acta Endocrinol* (*Copenh*) 79:709–719, 1975
37. Ogren L, Talamantes F: Prolactins of pregnancy and their cellular source. *Int Rev Cytol* 112:1–65, 1988
38. Landgraf R, Landraf-Leurs MM, Weissmann A, Horl R, von Werder K, Scriba PC: Prolactin: a diabetogenic hormone. *Diabetologia* 13:99–104, 1977
39. Ben-Jonathan N, Hnasko R: Dopamine as a prolactin (PRL) inhibitor. *Endocr Rev* 22:724–763, 2001
40. Barzilai N, Banerjee S, Hawkins M, Chen W, Rossetti L: Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. *J Clin Invest* 101:1353–1361, 1998
41. McCay CM, Crowell MF, Maynard LA: The effect of retarded growth upon the length of life span and upon the ultimate body size: 1935. *Nutrition* 5:155–171 [discussion 172], 1989
42. Han ES, Evans TR, Lee S, Nelson JF: Food restriction differentially affects pituitary hormone mRNAs throughout the adult life span of male F344 rats. *J Nutr* 131:1687–1693, 2001
43. Klebanov S, Diais S, Stavinoha WB, Suh Y, Nelson JF: Hyperadrenocorticism, attenuated inflammation, and the life-prolonging action of food restriction in mice. *J Gerontol A Biol Sci Med Sci* 50:B79–B82, 1995
44. Camper SA, Saunders TL, Katz RW, Reeves RH: The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. *Genomics* 8:586–590, 1990
45. Herlihy JT, Stacy C, Bertrand HA: Long-term food restriction depresses serum thyroid hormone concentrations in the rat. *Mech Ageing Dev* 53:9–16, 1990
46. Hsieh CC, DeFord JH, Flurkey K, Harrison DE, Papaconstantinou J: Effects of the Pit1 mutation on the insulin signaling pathway: implications on the longevity of the long-lived Snell dwarf mouse. *Mech Ageing Dev* 123:1245–1255, 2002
47. Hsieh CC, DeFord JH, Flurkey K, Harrison DE, Papaconstantinou J: Implications for the insulin signaling pathway in Snell dwarf mouse longevity: a similarity with the *C. elegans* longevity paradigm. *Mech Ageing Dev* 123:1229–1244, 2002
48. Flurkey K, Papaconstantinou J, Harrison DE: The Snell dwarf mutation Pit1(dw) can increase life span in mice. *Mech Ageing Dev* 123:121–130, 2002
49. Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Nagai R, Kimura S, Kadowaki T, Noda T: Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem* 277:25863–25866, 2002
50. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y: Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8:731–737, 2002

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/2/268/660361/db0203000268.pdf by guest on 03 March 2024